Determining Total Collagen Content: Stain Comparison

B-CHP vs. Masson's Trichrome vs. Picrosirius Red vs. Herovici's Stain

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Introduction

Histology and immunohistochemistry have been extremely helpful for clinical diagnosis and staging of numerous diseases including cancer and fibrosis. Scientists and pathologists have relied on a wide array of tissue stains, such as Masson's Trichrome, to help identify important anatomical features within tissue biopsies and enable them to make accurate diagnoses.[1,2] In idiopathic pulmonary fibrosis (IPF), for example, when non-invasive imaging modalities such as high-resolution computed tomography fail to distinguish the disease, clinicians turn to histology as the conclusive factor for diagnosis.[3,4] However, an experienced pathologist with a practiced eye must make decisions based on subtle differences in color, leading to inter- and intra-observer sampling variability.[1] Thus, a reliable, sensitive, and easy to read staining procedure can reduce errors by removing the human variability associated with subjective scoring.

A key factor for assessing fibrotic disease progression is determining the total collagen content (TCC) in a histology sample. The most common stains for assessing collagen content in tissue sections are Picrosirius Red (PSR), Masson's Trichrome (MT), Herovici's Stain, or a cocktail of collagen I & III antibodies. These stains are effective at monitoring total collagen content and can often inform pathologists about how much fibrotic scarring appears in tissues. However, a major drawback of these stains is they cannot discern between intact and damaged collagen.[5,6]

Collagen hybridizing peptides (CHPs) are proven to specifically recognize denatured collagen molecules in a variety of tissues, species, and disease models.[7] CHPs can identify damage in all 28 subtypes of collagen within the collagen superfamily, regardless of species or tissue type. By utilizing CHPs, pathologists will not only be able to assess TCC but also the fraction of active remodeling collagen (ARC), or denatured collagen, in a sample caused by disease or injury. Measuring the ratio of ARC to TCC can provide a prognostic value of how aggressively the fibrotic condition is progressing. CHP staining can easily determine this ratio, but no other stain or probe on the market can provide such information.

Here we report a direct stain comparison study using the aforementioned common collagen stains in order to detect damaged collagen in a fibrotic mouse model and compare them with the biotin labeled, B-CHP. All staining and image analysis was performed by HistoTox Labs, a third-party contract research organization, in order to prevent bias in evaluation and ensure all stains were carried out correctly. The severity of fibrosis in each slide was scored by an veterinary pathologist. The images in **Figure 1**, are representative images for evaluating TCC. Therefore, the sections stained by CHPs underwent heat-induced epitope retrieval (HIER) prior to staining to fully denature the collagen so it could be identified.

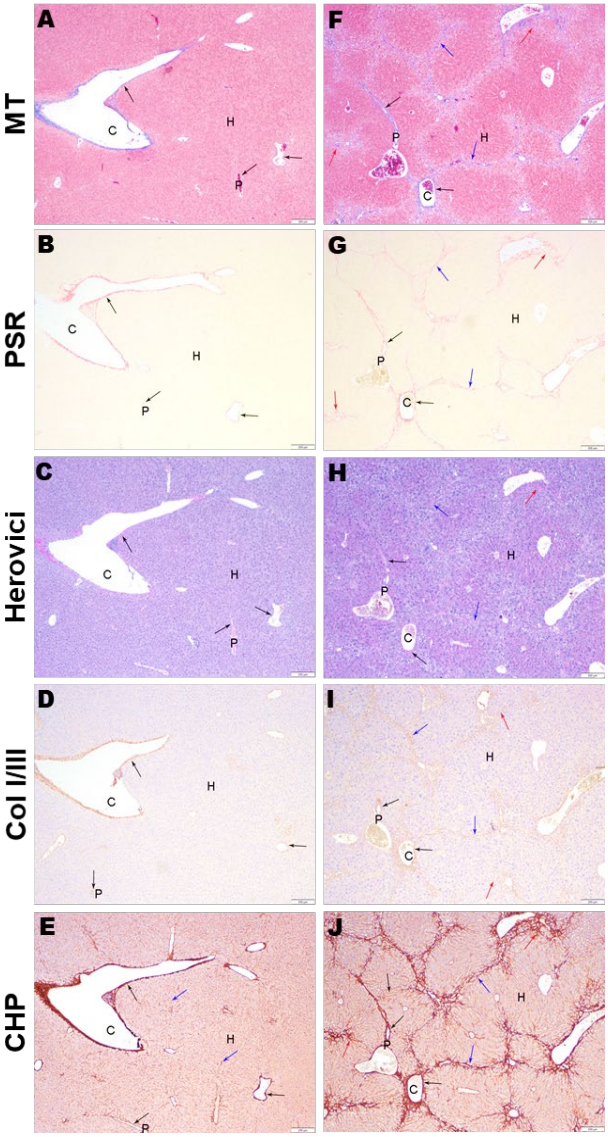

Figure 1. Representative photomicrographs of mouse livers stained using five different methods. Serial sections were taken from a healthy (control) mouse liver **(A-E)** and or a fibrotic mouse liver 8 weeks after injection with CCl_4 **(F-J)**. Collagen, identified by arrows in all photos, is stained blue in MT, pink/red in PSR, pink/red for mature collagen and blue for young collagen in Herovici's, dark to light brown for Col I/III cocktail, and dark brown in CHP staining. Other features of interest are labeled as: C-central vein, H-hepatocytes, P-portal triads. Magnification was 40X, scale bar = 200 µM.

Results

In this study, B-CHP performed just as well, if not better, than all other stains. Comparing the spatial staining pattern of CHP and all other stains, including antibody Col I/III cocktail, CHPs allowed easier visualization of damaged collagen and pathologists were able to easily identify damaged collagen without needing to interpret shades of color. Moreover, B-CHP was the only stain that identified reticulin fibers, indicating CHPs may also be used in place of silver stains for reticular fibers (e.g., Gomori's reticulin stain, Gordon and Sweet's reticulin stain, or Movat Pentachrome stain).[8] The reticulin stain is most useful for identifying changes to the hepatic architecture (loss of hepatocytes, thickening of hepatic cords, changes in lobulation, fibrosis/cirrhosis etc.). Some sources indicate it is a useful stain for diagnosing specific types of mesenchymal cell tumors and bone marrow fibrosis.[9]

Pathologist's Note:

"I thought the CHPs were very consistent and easy to read. It highlighted more collagen than any of the other stains and made it a lot easier to identify dissecting fibrosis between hepatocytes. Even with some degree of background staining (likely due to our current Ab dilution), the CHPs were good at highlighting fibers and maintaining the collagen morphology, allowing for better identification of real vs non-specific staining. In livers we typically use PSR or Col I/III, both of which have a two-tone color spectrum (red/yellow or chromogenic IHC). The fact that CHPs are biotinylated to allow for chromogenic IHC is a plus, as this allows for good contrast between collagen and non-collagen (unlike Masson's trichrome and Herovici's). Overall, I thought the stain performed better for collagen detection, seemed to have a consistent staining pattern, and was easy to interpret."

Automated Image Analysis

In addition to standard histology assessment, HistoTox Labs assessed each of the slides using automated image analysis to quantify the area of collagen stained in each sample. For all images, regions of interest were generated to include liver tissue, but exclude artifacts (folds, tears, etc.) large blood vessels, and non-liver tissue. Regions of interest were then subjected to several imaging filters to separate positively stained areas from negative areas. The positive area was quantified, then compared to the total area in the region of interest.

In PSR staining, collagen appears bright red and remaining tissue a pale yellow. These images were easily characterized using automated image analysis without issue due to the high contrast between positive and negative regions. In Masson's Trichrome, collagen appears blue, and cells appear red. However, some endothelial cells exhibited weak blue staining in their cytoplasm and as a result, were intermittently detected by the automated image analysis even after careful thresholding. This likely resulted in an over-detection of collagen during quantification. Herovici-stained sections exhibited pink/red staining for mature collagen and pale blue for young collagen. Hepatocyte cytoplasm was stained a pale pink to pink-purple and mineral regions were stained dark purple to black. Overall, contrast was very-poor for Herovici-stained tissue, and as a result, the image analysis could only be performed for the mature collagen (pink/red) as the young collagen could not be differentiated from the negatively stained region.

CHP-stained sections exhibited light brown staining of collagen fibers (DAB staining of B-CHP). Negative regions were stained light blue to grey for cytoplasm and dark blue for nuclei (due to hematoxylin counter-stain). Image analysis was generally specific to CHP-labeled collagen fibrils, but some areas of elevated background staining required the algorithm to slightly under-detect CHP-stained regions.

Collagen was detected in all samples and with all stains, but the quantity of collagen varied (**Figure 2**). In naïve samples the total collagen was low across all samples and staining methods. However, substantial differences were observed between stains in samples from diseased tissue. The most collagen was detected using Masson's Trichrome. However, as noted above, the weak blue staining of hepatocyte cytoplasm interfered with specific analysis, and the quantity of collagen is likely overrepresented. HistoTox Labs noted: "True staining quantification is likely more similar to PSR detection." PSR and CHP with HIER were highly similar and likely represent an accurate assessment of total collagen content. Herovici staining was difficult to accurately assess using image analysis and had the smallest area of detected collagen. In addition, consistent with CHP's specificity for denatured collagen vs all collagen, tissue sections that did not undergo HIER had a lower amount of collagen detected, indicating that both intact and denatured collagen are elevated in the fibrotic tissue. Representative images and analysis from fibrotic samples are shown in **Figure 3**.

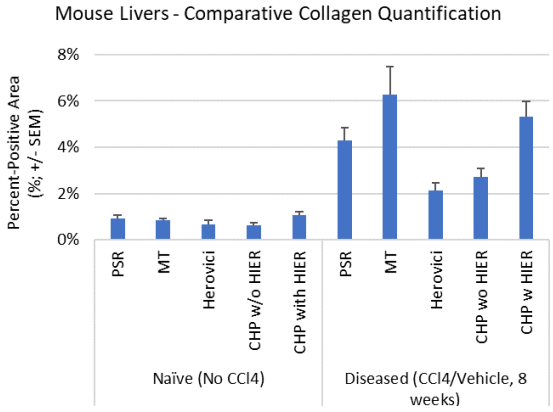

Figure 2. Collagen quantification by image analysis. Collagen was detected in all samples, with low overall collagen detected in naïve samples, and significantly higher quantities of collagen detected in diseased samples.

Summary

CHP staining of fibrotic liver tissue performed just

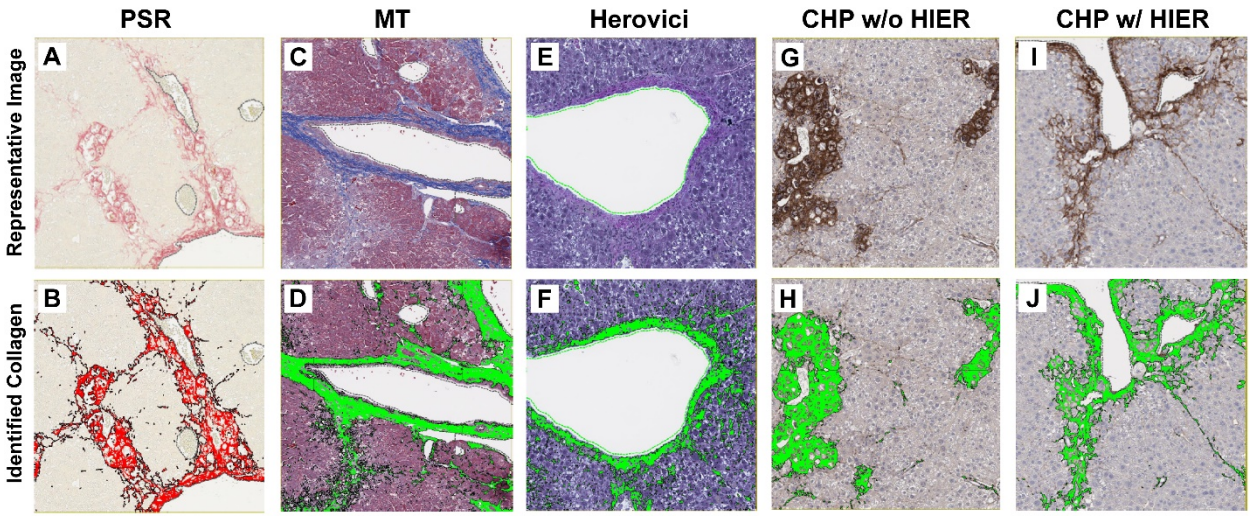

Figure 3. Representative micrographs of detected collagen in fibrotic liver samples (CCl_4, 8 weeks) using automated image analysis. In all images, green dotted lines represent the outer edge of the tissue and black dotted lines indicate areas excluded from analysis. Images in the top row are the images prior to analysis, and those in the bottom row show collagen highlighted by the image analysis (Red for PSR, bright green for all others).

as well if not better than other common stains for detecting collagen in tissue sections. Expert pathologists noted that CHP staining "performed better for collagen detection [than other stains], seemed to have a consistent staining pattern, and was easy to interpret." In automated image analysis, CHP staining quantified collagen area as accurately as PSR, and more accurately than Masson's Trichrome, and Herovici's stain. Overall, CHP is a one-step collagen stain that has high contrast and is easier to read than other collagen histology stains, while maintaining high accuracy.

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Materials & Methods

Liver fibrosis was developed by administering carbon tetrachloride, CCl_4, to mice before sacrificing them at 8 weeks and sectioning their livers. As a control, healthy mice which were not given CCl_4. Each stain was run in sets of five at every severity level indicated.

To obtain the ratio between damaged collagen and total collagen for prognostic applications, one set of sections was purposefully heat-denatured via standard heat-induced epitope retrieval (HIER) methods. This would serve as the total collagen content; a serial section was then stained without undergoing HIER and then both sections were evaluated using Visiopharm software for image analysis to get quantitative values for each. Below are the staining methods that were used.

Masson Trichrome Procedure:

1. Deparaffinize slides and hydrate to distilled water.
2. Mordant slides in Bouin's overnight at room temperature.
3. Remove slides from Bouin's and rinse thoroughly in running tap water until tissue is colorless.
4. Place slides into Weigert's Working Solution for 5 minutes.
5. Rinse slides in three changes of running tap water.
6. Place slides into Biebrich Scarlet Acid Fuchsin for 15 minutes.
7. Rinse slides in three changes of running tap water
8. Place slides into PPA for 12.5 minutes.
9. *Without rinsing*, place slides into Aniline Blue for 7.5 minutes.
10. Rinse slides in three changes of running tap water.
11. Place slides into 1% Acetic Acid for 5 minutes.
12. Dehydrate slides.
13. Coverslip slides with permanent mounting media.

Picrosirius Red (PSR) Procedure:

1. Deparaffinize slides and hydrate to distilled water.
2. Mordant slides in Bouin's overnight at room temperature.
3. Remove slides from Bouin's and rinse thoroughly in running tap water until tissue is colorless.
4. Place slides into Picrosirius Red Working Solution for 15 minutes.
5. Rinse slides in three changes of running tap water.
6. Place slides into Picric Acid for 40 minutes.
7. Rinse slides in tap water for 2 minutes.
8. Dehydrate slides.
9. Coverslip slides with permanent mounting media.

Herovici's Procedure:

1. Deparaffinize slides and hydrate to distilled

water.
2. Place slides in filtered Weigert's hematoxylin for 5 minutes.
3. Rinse in tap water for 45 seconds.
4. Place slides in Herovici's working solution for 2 minutes.
5. Without rinsing, immerse slides in 1% acetic acid for 2 minutes.
6. Dehydrate slides.
7. Coverslip slides with permanent mounting media.

Collagen Type I and Type III Cocktail (Col I/III) IHC Procedure:

1. Perform antigen retrieval by heating slides in a sodium citrate solution (pH = 6) for 25 minutes.
2. Wash slides using Bond™ Wash Solution (Leica Biosystems) for 5 minutes.
3. Incubate slides in antibody solutions for 30 minutes (Coll I- 1:400, PB9939 Booster Bio & Coll III- 1:300 22734-1-AP ProteinTech).
4. Wash slides using Bond™ Wash Solution for 5 minutes.
5. Detect antibody binding using an HRP-conjugated secondary polymer (Secondary antibody conjugated to HRP-polymer backbone).
6. Visualize slides using SignalStain® DAB Substrate Kit (Cell Signaling Technology) for antibodies and a Hematoxylin counterstain to visualize nuclei.
7. Dehydrate slides.
8. Coverslip slides with permanent mounting media.

Collagen Hybridizing Peptide (CHP) with HIER Procedure:

1. Bake slides in 60 °C oven for 1-2 hours.
2. Pretreat and block slides using the following steps. Wash slides for 5 min between each step using Bond™ Wash Solution (Leica Biosystems)
   a. Dewax at 72 °C.
   b. Perform heat retrieval with Leica ER2 (pH 9 EDTA-based HIER solution) for either 25 min at 94 °C or 2 hours at 70 °C.
   c. 3% H_2O_2.
   d. Avidin Block.
   e. Biotin Block.
3. Incubate with B-CHP.
   a. Place slides in PBS with Tween (PBS-T) until peptide is ready for application.
   b. Dilute B-CHP to 15 µM in PBS-T and incubate in an 80 °C water bath for 10 minutes.
   c. While heating, remove PBS-T from slides and encircle tissue with a wax pen.
   d. Remove B-CHP solution from water bath, place on ice for ~30 seconds, then promptly apply ~250 µL to each slide.
   e. Incubate in a humidity chamber at 4 °C overnight.
4. Wash slides for 5 min with Bond™ Wash Solution.
5. Apply 250 µL of ABC HRP Reagent (Vector) at RT for 60 minutes.
6. Wash slides with Bond™ Wash Solution.
7. Apply 250 L of Signal Stain DAB (Cell signaling) at RT for 5 min.
8. Rinse with diH_2O three times.
9. Counterstain with Hematoxylin, dehydrate with graded alcohols, and clear in xylene.
10. Coverslip slides with permanent mounting media.

Collagen Hybridizing Peptide (CHP) with no HIER Procedure:

1. Dewax and rehydrate slides using a standard histology autostainer at RT.
2. Pretreat and block slides using the following steps. Wash slides for 5 min between each step using Bond™ Wash Solution (Leica Biosystems).
   a. 3% H_2O_2.
   b. Avidin Block.
   c. Biotin Block.
3. Stain slides with B-CHP following steps 3-10 from CHP with HIER procedure above.

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