

Quantifying Collagen Content in Histopathology by Fluorescent CHP Hybridization

Dr. Lucas Bennink and Dr. Yang Li
3Helix Inc. Salt Lake City, Utah, USA. 84108

Note: CHPs are a research use only (RUO) reagent, and not intended to be used as a diagnostic.

Introduction

The fields of histology and immunohistochemistry have been extremely helpful for clinical diagnosis and staging of numerous diseases such as cancer and fibrosis. Scientists and clinicians have relied on having a wide array of tissue stains to help identify important anatomical features and aspects within the tissue biopsies that enables them to make an accurate diagnosis.^{1,2} However, an experienced pathologist with a practiced eye must make decisions based on subtle differences in the staining, such as Masson's Trichrome, which leads 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. Determining the collagen content in a histology sample is the key for assessing fibrotic disease progression. Collagen hybridizing peptides (CHPs) have proven to be a unique tool for specifically recognizing denatured collagen molecules in a variety of tissues, species, and disease models.³ Here, we describe a fluorescent image quantification protocol for use with Image-J/FIJI that can be used to objectively measure collagen content in a histology slide. This measurement is based on CHP fluorescent intensity within the tissue sample, which **has been readily treated to completely denature its collagen content on purpose**. This protocol is designed to work with formalin-fixed paraffin-embedded (FFPE) that have undergone heat-mediated antigen retrieval as well as on frozen tissue sections.

Materials & Methods

Total Collagen Content

To use CHPs for evaluating the total collagen content within a FFPE tissue section, the collagen must be fully denatured to allow for CHP binding on all available collagen. We recommend heating the tissue section to thermally denature the collagen. Our tests indicate that the long heating periods used in heat induced epitope retrieval (HIER) methods are sufficient to completely denature the collagen in the sample (regardless of the buffer used). The tissue sections shown in this application note were placed in 50 mL of citrate buffer in a tissue steamer for 45 minutes at 95-100 °C. Alternatively, to denature the collagen content in a frozen tissue slide, a water bath can be used to heat up a sealed 50 mL tube containing DI water to temperatures over 85 °C. After heating, pipette the hot DI water onto the tissue samples and allow to sit for 5 minutes (repeat 5X).

Experimental Protocol

1. FFPE Sections: perform deparaffinization prior to CHP staining by submerging sections for 2 x 5-minute washes in xylenes, 100% ethanol, 95% ethanol, 50% ethanol, and DI water in this order.
2. Perform HIER or purposefully heat denature the tissue for the determination of total collagen content.

3. Create a working solution for CHP staining by dissolving CHP powder in 1x PBS so that the concentration is within a 5-20 μ M range. The exact concentration depends on the optimized parameters for the tissue section and the volume needed.

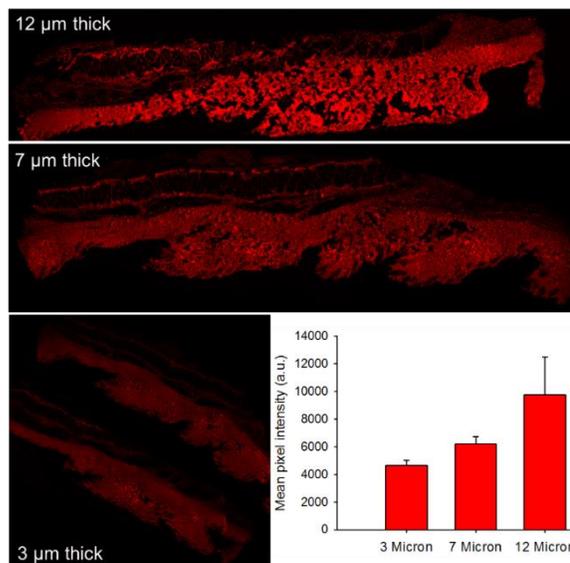


Figure 1. Normal rabbit skin sections of different thickness stained with 20 μ M R-CHP after heat induced epitope retrieval. As the thickness of the tissue sections increase, more CHP intensity is observed. Signal quantification using ImageJ/FIJI shows a linear correlation between tissue thickness and signal intensity.

4. Heat CHP solution to 80 °C. Since CHP can self-assemble into homotrimers in solution over time (e.g., during storage at 4 °C) and lose its driving force for collagen hybridization, the trimeric CHP needs to be thermally dissociated to single strands by heating briefly at 80 °C.
5. Quickly quench hot CHP solution in an ice water bath (~30 sec) before using it to bind to unfolded collagen in the tissue.
6. Apply quenched CHP solution to tissue section. Completely cover tissue section with CHP staining solution and allow overnight binding at 4 °C in a humidity chamber.
7. Wash sections using 3 x 5-minute washes of 1x PBS or 1x Tween-20.
8. Mount coverslips and image.

For a detailed protocol on tissue preparation please see our [User Manual](#).

After washing off excess CHP solution, the tissue can be imaged, and imported into ImageJ/FIJI for signal quantification (**See below**). The signal intensity correlates with the amount of collagen within the sample. As the tissue sections get thicker, they contain more collagen and therefore we expect to see higher signal intensity from

CHP binding to denatured collagen strands. In Figure 1, CHP signal is more intense as the tissue gets thicker, confirming that CHP signal correlates to total collagen content. The intensity was determined by measuring the average pixel intensity of the total area imaged after background subtraction. This method will allow researchers to easily visualize and quantify the total collagen content within a tissue section.

Discussion

Once the appropriate imaging settings are found, CHP staining allows for easy determination of total collagen content in different tissue samples. This is valuable for examining total collagen content in fibrotic tissues, and it may prove beneficial for evaluating wound healing or disease progression as collagen remodeling is associated with numerous pathological disease and healthy organ upkeep. The fluorescent staining can be quantified using the ImageJ/FIJI platform.

Instrument Parameters

Instrument: EVOS Auto-FL Imager (LifeTechnologies)

Detection: Fluorescence (R-CHP: 548/563 nm; F-CHP: 494/512 nm)

Magnification: 4X up to 40X (images in this application note were taken at 4X)

Light Settings: Choose an acceptable exposure time, light brightness, and gain and apply these setting to all sections imaged. Ensure that images do not contain saturated pixels, adjust settings accordingly.

Image-J/FIJI Macro Steps

Please follow the steps below to complete the image analysis correctly. The comments describe what each step of the macro code does, they are shown in green. We have also provided the code without comments so that you can copy and paste it directly into ImageJ by going to Plugins-> New-> Macro. **NOTE:** The user will need to determine an acceptable threshold value to help remove some of the background auto-fluorescence. This macro works best with high signal-to-noise ratio, if there is low signal with lots of background noise or auto-fluorescence, you will need to include additional steps prior to running this macro.

```
setBatchMode(true); //Tells imageJ that the following code will be
run on a batch of files.
output = getDirectory("Output Directory"); //sets the output
variable as the user defined directory with title "output
Directory". ImageJ will ask user to specify what folder to
output data into. ImageJ will ask for the output
directory FIRST.
input = getDirectory("Input Directory"); //sets the input variable
as the user defined directory with title "Input
Directory". ImageJ will ask user to specify what folder to
search through. It is easier if you place all the images
you wish to evaluate in the same folder.
Dialog.create("File Type");
Dialog.addString("File Suffix: ", ".tif"); //tells ImageJ to only use
files of type ".tif". Users can specify what file type they
wish ImageJ to use (e.g. ".jpeg").
suffix = Dialog.getString();

processFolder(input); //This is how ImageJ will search through the
user specified input folder.
function processFolder(input){
list=getFileList(input); //Sets the variable list to the
```

number of files in the input folder.

```
for(i=0;i<list.length;i++){
if(File.isDirectory(input+list[i]))
processFolder(""+input + list[i]);
if(endsWith(list[i],suffix))
processFile(input, output, list[i]); //This loop
scans the folders/subfolders/files to locate the files with
the correct suffix.
}
}
//This is the process that will act on each image file in the folder
function processFile(input, output, file) {
open(file); //Opens file of type ".tif".
print("Processing: " +input+ file); //Prints "Processing:
input (file path) and file (filename) to the log.
run("16-bit"); //Converts image to 16-bit. To save time,
you may want to run the code from this line onwards as
a test to see if any further image processing is needed. It
will just perform the steps on a single image that is
already open and selected in ImageJ.
run("mpl-iridis"); //Sets the image to LUT mpl-iridis
which is useful to view pixel intensity.
run("Measure"); //Measures the pixels in the image
run("Set Measurements...", "area mean standard min
integrated limit display redirect=None decimal=3");
//Allows user to define what measurements are taken
during the "Measure" Command. Make sure that "area,
standard deviation, min & max gray value, integrated
density, mean gray value, limit to threshold, and display
label" are all checked to be measured. You can double
check this by going into Analyze-> Set Measurements.
setAutoThreshold("Mean dark"); //The auto-threshold
method is set to mean with the dark background option
selected.
setThreshold(11,255); //Set Threshold for threshold
based on positive control, this can be changed by
clicking Image->Adjust->Threshold and choosing the
value that shows all the signal but cuts out any
background. ***IMPORTANT-you need to set the
threshold to a value that you choose based on the
fluorescent intensity of your positive control. To do this
simply go to Image-> Adjust->Threshold, select Mean
from the threshold-type dropdown menu and select
dark background. You can adjust the min and max
threshold values and then hit set. Do not hit apply, as
this will create a mask and change all pixels with a value
above the threshold to 255 (red).
getThreshold(lower, upper); //Finds the upper and
lower limit of threshold, this should match the values
you set in the step above.
print("Min: "+lower+ " - "+Max: "+upper); //Prints the
min and max threshold values to log.
run("Measure"); //Runs measure again.
title = getTitle(); //Sets the variable title to be the title of
the file, i.e. it fetches the filename.
saveAs("jpeg", output+title); //Saves the file as a .jpeg,
to the user defined output folder with the filename.
print("Saved to: "+output); //Prints "Saved to: the
output filepath" to the log.
}
selectWindow("Results"); //Selects the window titled "Results",
which is where all the measurements data is stored.
saveAs("Results", output+ "Results"+ ".tsv"); //Saves the data from
the "Results" table to your defined output folder as
 filetype .tsv (easy to copy and paste into excel) with the
name Results.
```

```
print("Task Complete");//Prints "Task Complete" to the log to
    indicate that ImageJ has finished searching the folder,
    and processing all the images with the defined filetype
    in the folder.
```

This process was designed based of previous literature that focused on the ImageJ/FIJI image quantification tools and methods.⁴⁻⁶ We want to provide the most effective and efficient protocol for our customers. As such, we ask that if you have optimized these steps or found an alternative tool within ImageJ/FIJI that works better, please contact us at: info@3helix.com and we will update this application note and acknowledge your contribution!

Note: CHPs are a research use only (RUO) reagent, and not intended to be used as a diagnostic.

References

- (1) Weiskirchen, R.; Weiskirchen, S.; Tacke, F. Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications. *Mol. Aspects Med.* **2019**, 65 (June 2018), 2–15.
- (2) Goodman, Z. D. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J. Hepatol.* **2007**, 47, 598–607.
- (3) Hwang, J.; Huang, Y.; Burwell, T. J.; Peterson, N. C.; Connor, J.; Weiss, S. J.; Yu, S. M.; Li, Y. In situ imaging of tissue remodeling with collagen hybridizing peptides. *ACS Nano* **2017**, 11, 9825–9835.
- (4) Bankhead, P. Analyzing fluorescence microscopy images with ImageJ. **2014**, No. May.
- (5) Jensen, E. C. Quantitative Analysis of Histological Staining and Fluorescence Using ImageJ. *Anat. Rec.* **2013**, 296, 378–381.
- (6) Miura, K. Python + ImageJ, FIJI Cookbook http://wiki.cmci.info/documents/120206pyip_cooking/python_imagej_cookbook (accessed Dec 2, 2019).

```
setBatchMode(true);
output = getDirectory("Output Directory");
input = getDirectory("Input Directory");
Dialog.create("File Type");
Dialog.addString("File Suffix: ", ".tif");
suffix = Dialog.getString();

processFolder(input);
function processFolder(input){
    list=getFileList(input);
    for(i=0;i<list.length;i++){
        if(File.isDirectory(input+list[i]))
            processFolder(""+input + list[i]);
        if(endsWith(list[i],suffix))
            processFile(input, output, list[i]);
    }
}
function processFile(input, output, file) {
    open(file);
    print("Processing: " +input + file);
    run("16-bit");
    run("mpl-iridis");
    run("Measure");
    run("Set Measurements...", "area mean standard min
integrated limit display redirect=None decimal=3");
    setAutoThreshold("Mean dark");
    setThreshold(11,255);
    getThreshold(lower, upper);
    print("Min: "+lower+ " - "+Max: "+upper);
    run("Measure");
    title = getTitle();
    saveAs("jpeg", output+title);
    print("Saved to: "+output);
}
selectWindow("Results");
saveAs("Results", output+ "Results"+" .tsv");
print("Task Complete");
```

Copy and paste the code below into ImageJ