

Image J Analysis for Collagen Area Fraction of Picrosirius Red Stained Tissue

Description

Here we will quantify the amount of collagen in a given area of myocardial tissue, i.e. the collagen area fraction. For this protocol we are using a Picrosirius Red stain which binds specifically to collagen fibrils of varying diameter. It is recommended to use high quality images (i.e. .tiff) rather than compressed formats (i.e. .jpeg). Image J will be the software of choice for the analysis.

The following steps below will describe the manual process of measuring collagen area fraction. An automated macro written for Image J is listed below as well. NOTE: automated thresholding may produce overestimates of the collagen area fraction, please be cautious when using. Also, this macro assumes that there are no open spaces (i.e. tissue area equals total area) in the image.

Overview

- i. Load Image
 - A. Adjust image scale to physical units (i.e. μm) **OPTIONAL!!**
 - B. Convert RGB image to grayscale
 - C. Segment (intensity threshold) red-stained collagen
 - D. Measure the area of the thresholded collagen
 - E. Segment the tissue from the background (i.e. white space) **OPTIONAL!!**
 - F. Measure the total segmented tissue area **OPTIONAL!!**
 - G. Compute collagen area fraction

Methods

(i) Load Image *File > Open*

(A) Set Scale **OPTIONAL!!**

Typically your image dimensions will be in pixels; in order to set the size of the Image in physical units you must calibrate the image. This is typically done by using a calibration slide (e.g. a slide that has a labeled grid in microns)

To set the scale in Image J

1. Use the line selection tool and draw a line corresponding to the known grid size.
2. Select \rightarrow *Analyze > Set Scale*

3. Notice the “Distance in Pixels” is already filled in; this is the distance that you measured in Step A.1.
4. Enter the known distance (e.g. 1.00)
5. Enter the Pixel Aspect Ration (i.e. 1 for a square pixel)
6. Enter the physical units of the known distance (i.e. cm, mm)
Note: to enter microns use “um”
7. Click OK

(B) Convert image to Grayscale

Image J uses an intensity threshold and therefore requires that your images be in grayscale (i.e. for an 8 bit file each pixel contains an intensity value between 0 – 255)

To convert the current image to grayscale

1. Select *Image > Type > RGB Stack*
2. You will now see 3 grayscale images in a stack (first-red, second-green, third –blue)
3. Notice that the green channel has the best contrast between tissue and stain

Note: To view all three channels in one image (i.e. a montage) select *Image > Stacks > Make Montage* click *Labels* in the dialog window to display channel label (i.e. red, green, blue)

(C) Segment collagen from tissue

Here we will use the green channel to segment the red stained collagen (dark intensity) from the tissue (light intensity)

To threshold the image

1. Move horizontal bar on RGB stack window so that the Green channel is viewed
2. Select *Image > Adjust > Threshold*
Note: Image J will by default color the segmented pixels in red

3. Image J will automatically set the threshold level; manually adjust this level by moving the bottom horizontal bar in the “Threshold” window from left to right.

4. Once satisfied with segmented area, Click “set”

Note: Ensure that the lower threshold is zero and the upper will default to the value you manually determined from the bottom horizontal bar.

Note: To remove thresholding click the “Reset” button in the “Threshold” window

Note: To view all three channels in one image (i.e. a montage)

select *Image > Stacks > Make Montage*

click *Labels* in the dialog window to display channel label (i.e. red, green, blue)

(D) Measure area of collagen stained tissue

We are now ready to measure the area of the Picrosirius Red stained collagen

First we will set our measurements

1. Select *Analyze > Set Measurements*

2. In the popup window make sure the following are checked: Area, Area Fraction, Limit to Threshold, Display Label

Note: In the case where there are no “gaps” (e.g. tissue on white background) the measure of “Area Fraction” will tell you directly the relative amount of collagen area to total tissue area. **For this case skip E and F.**

However, in the case where open spaces occur (e.g. short axis slice of the heart with LV and RV will contain white background in the cavity space), the measure of “Area Fraction” will underestimate the true collagen area fraction. **In this case do steps E and F.**

Now we can proceed to take our measurements

3. Press the “m” key on the keyboard, the “Results” window will popup with your current measurement.

(E) Segment total tissue area from background **OPTIONAL!!**

We will repeat Steps C.1 – C.4 but for the entire tissue

(F) Measure the total tissue area **OPTIONAL!!**

We will repeat Steps D.1 – C.3 but for the entire tissue

G) Compute collagen area fraction (CAF)

We are now ready to compute the collagen area fraction

I will list two methods

1. In the case where there are no spaces between tissue:
CAF = “area fraction” measured from (D)
2. In the case where there are spaces between tissue
Collagen area (CA) = “area” measured from (D)
Tissue area (TA) = “area” measured from (F)
CAF = CA/TA * 100%

Automated Thresholding Macro

```
// CalculateAreaFraction.txt
// Quantification of Collagen Area Fraction from PicroSirius Red
// Load Image first

// ----- Macro Starts Here -----//

// convert to RGB Stack, Select green channel for highest contrast
run("RGB Stack");
setSlice(2);

// Optional !! Set image scale, this requires that you have a calibration
//image, Inputs: known "distance" (in pixels), known distance "known" (in
//microns), pixel aspect ratio (typically 1) "pixel", and units "unit"
run("Set Scale...", "distance=317 known=200 pixel=1 unit=um");

// Automatic Threshold
setAutoThreshold();
getThreshold(min, max)
setThreshold(0, max);

// Area Fraction Measurement
```

```
run("Set Measurements...", "area_fraction limit display redirect=None  
decimal=3");  
run("Measure");  
selectWindow("Results");  
  
// End Macro
```