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Picrosirius Red (PSR) Staining and Quantification in Mouse Ovaries

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We use this protocol and it's working

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Abstract

Picrosirius Red (PSR) staining is used to visualize collagen fibers in various tissues. When PSR binds to collagen fibers (specifically collagen I and III), it results in a dark red stain whereas the cytoplasmic regions appear yellow or faintly pink [1, 2]. Generally, ovarian non-fibrotic regions stain darker pink than liver non-fibrotic regions, possibly due to the difference in liver and ovarian tissue composition. Young adult females exhibit minimal PSR staining, which increases to distinct foci in mid-to-advanced reproductive-age animals, and eventually manifests throughout the stroma in the oldest animals [3]. This protocol is designed for mouse ovaries. Due to the higher collagen content in human, non-human primate, cow, and pig tissues, modifications will likely be needed to adapt it for those species.

*Note: Original mouse ovary PSR staining protocol developed by the Duncan lab as a modification of the protocol from the Pritchard lab. Original Publication: Briley et al. Reproduction 152(3): 245 - 260, 2016 (**https://doi.org/10.1530/REP-16-0129**). This protocol was prepared by Dr. Soygur with permission and approval from Dr. Pritchard and Dr. Duncan.*

Materials

1. Deionized (DI) water *(CAS 7731-18-5)*
2. 1X Phosphate Buffered Saline (PBS) *(CAS 7758-11-4)*
3. Modified Davidsons Fixative **Electron Microscopy Sciences Catalog #**64133-50
4. Citrisolv Clearing Agent **Fisher Scientific Catalog #**22-143-975
5. Sirius Red (Direct Red 80) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**365548
6. Picric acid solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P6744
7. Hydrochloric Acid Concentrate, 10N ACS (Certified) **Fisher Scientific Catalog #**SA49
8. Eppendorf™ Safe-Lock Tubes 1.5 mL - Microtube (Fisher Scientific Catalog #022363204)
9. Superfrost Microscope slides (Fisher Scientific, Catalog #12-550-15)
10. Microscope cover glasses (VWR 60×24 mm, No 1.5, Catalog #48393-251)
11. Eukitt Mounting Media **Electron Microscopy Sciences Catalog #**15320
12. Ethanol **Decon Labs Catalog #**2701G

Equipment

EZ-Quick Slide Staining Set	NAME
IHC World	BRAND
IW-2510	SKU
https://store.ihcworld.com/ez-quick-slide-staining-set/	LINK

Troubleshooting

Tissue Harvest and Embedding

1 Dissect mouse ovaries, transfer them into a petri dish containing PBS. Remove surrounding tissues under a dissection microscope.

2 To fix the samples, transfer them into 1.5 mL Eppendorf tubes containing 1 ml of Modified Davidson's fixative. Incubate them on an orbital or rocker shaker at room temperature for 2 hours (h) followed by overnight incubation at 4 °C.

3 The next day, wash them in 70% EtOH three times for 10 minutes (mins) each. For downstream tissue processing and embedding into paraffin, use automated tissue processors.

4 Section paraffin blocks at a thickness of 5 µm and collect sections on Superforst Microscope slides.

Preparing Solutions

5 Ethanol solutions, 30% and 70% Ethanol in DI water: Dilute appropriate volume of 100% Ethanol in DI water.

6 Making the Picrosirius Red (PSR) Solution: Combine 0.5 g of Direct Red 80 with 500 mL of 1.3% Picric Acid in an autoclaved beaker.

> Note
>
> Do not substitute Direct Red 80 with any other product, and ensure the picric acid is at the specified concentration. Otherwise, the staining will not be effective.

6.1 Stir on a stir plate without heat until the powder is combined into the liquid and forms a homogenous solution.

6.2 Transfer the picrosirius red solution to another autoclaved bottle for storage and wrap the bottle in tin foil to keep the PSR solution in the dark. The PSR solution can be kept for up to 12 years and reused multiple times.

> Note
>
> Pour PSR solution back into the original container for reuse as it is light-sensitive.

7 Making the Acidified Water Solution (0.05N HCl): Under a fume hood, add 5 mL of HCl (10N) to 995 mL of MilliQ water in an autoclaved bottle. Solution can be stored for ~3 months.

PSR Staining Protocol

8 Set up plastic slide staining containers for each solution/step (3 containers for Citrosolv, 3 containers for 100% Ethanol, 1 container for 70% Ethanol, 1 container for 30%Ethanol, 2 containers for DI water, 1 container for PSR solution, 1 container for HCl). Appropriate volume of each solution should be added to containers until each solution covers the tissue present on the slide. Always use fresh acidified water. Citrisolv and Ethanol can be re-used for up to a month and 1 week, respectively (if properly covered to prevent evaporation).

9 Place slides in a carrier.

10 Dip carrier vigorously in Citrisolv ten times and then incubate for 3 minutes. Repeat this for a total of three separate Citrisolv incubations, each time in a new container.

Note

Vigorous Dipping: Before each incubation, dip the slides 10 times, agitating the solution with carrier adequately without causing excessive splashing, as this may impact concentration and coverage.

Note

Ensure each solution is fully drained into its container before transferring the slides to a new solution. This is especially important when moving between solutions, as residual liquid may alter concentration, staining quality, or washing effectiveness.

11 Dip carrier vigorously ten times in 100% EtOH and then incubate for 1 minute. Repeat for a total of two times, each time in a new container.

12 Dip carrier vigorously ten times in 70% EtOH in DI water and then allow to sit for 1 minute.

13 Dip carrier vigorously ten times in 30% EtOH in DI water and then allow to sit for 1 minute.

14 Dip carrier vigorously ten times in DI water and then incubate for 1 minute. Repeat for a total of two incubations, each in a new container.

15 Dip carrier vigorously ten times in PSR solution and incubate for 40 minutes.

Note

PSR incubation time may range from 40 to 50 minutes.

16 Dip carrier vigorously ten times in the acidified water solution (0.05N HCl) and incubate for 90 seconds.

Note

HCl may cause over-destaining of the samples depending on the fixative used. HCl can be replaced with Acetic Acid if it is needed (add 5 ml of Acetic Acid to 995 ml of DI water in an autoclaved bottle. Incubate the slides in 0.5% Acetic Acid solution for 7 minutes and this wash can be repeated if necessary).

17 Tap carrier several times onto a paper towel to remove excess acidified water.

18 Dip carrier vigorously ten times in 100% EtOH and then incubate for 30 seconds. Repeat this for a total of three incubations, each time in a new container.

19 Dip carrier vigorously ten times in Citrisolv and incubate for 5 minutes.

20 Keep slides in Citrisolv until ready to coverslip. Do not allow samples to dry. Eukitt mounting media distributes best when slide is wet.

21 Dry off back of slide.

22 Pipette ~100 µL of mounting media on to slide.

23 Slowly tip the coverslip onto the mounting medium and avoid creating bubbles as you lower it into place.

Note

Allow the Eukitt mounting media to harden overnight before imaging.

Image Acquisition

24 Image slides at 20X magnification. Each ovary should be analyzed by examining at least three sections: one from the top third, one from the middle third, and one from the bottom third.

25 Add a scale bar to a place suitable in the file. Do not place on the tissue.

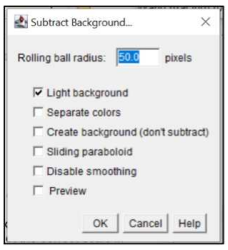
26 Set up aspreadsheet to record data. The spreadsheet should record: Ovary name, section of analysis, scale, total area of the tissue, total area of the PSR positive tissue.

	A	B	C	D	E	F
1	Ovary	section	Scale	total area	psr postive area	% area psr positive
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						

Analysis

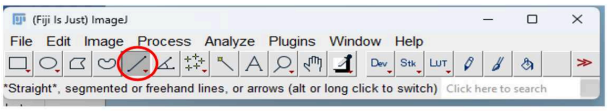
27 Open .tif fil in ImageJ. Click Process> Subtract Background.

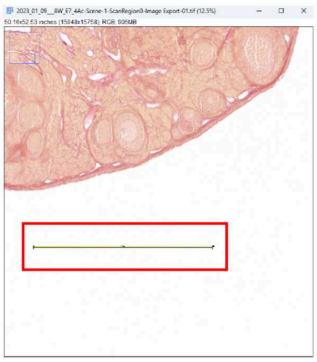
27.1 Ensure only ‘Light background’ is selected. Press >Ok



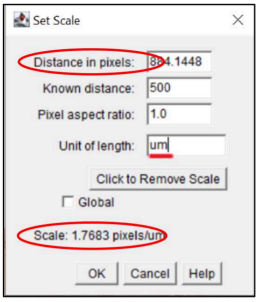
28 Set scale of the tissue.

28.1 In ImageJ select the 'Straight' line tool and draw a line the same length as the scale bar.



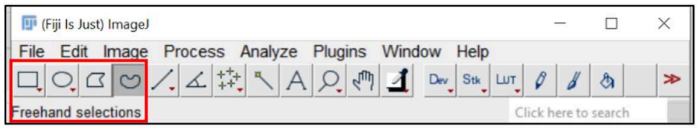


28.2 Once the line is drawn in ImageJ select Analyze> Set Scale. Type the known distance of the scale bar into the known distance box. Record the scale in the spreadsheet. Select Ok.

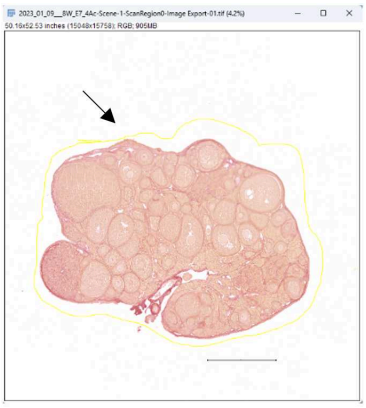


29 Select ovarian tissue. Exclude any extra-ovarian tissue and the scale bar.

29.1 Select ‘Freehand selections’.



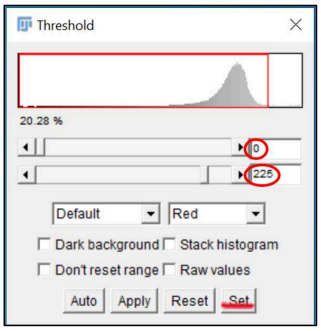
29.2 Draw around the ovary to only include ovarian tissue within the selection. This will ensure the analysis only includes ovarian tissue and not any surrounding tissues.

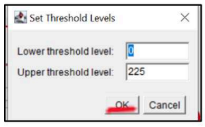


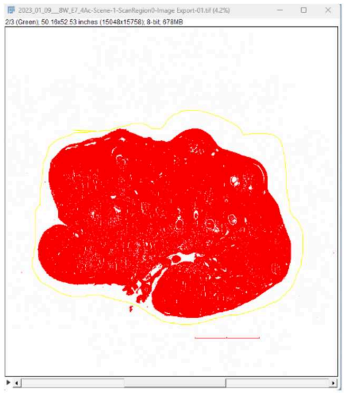
30 Select Image>Type>RGB stack. This splits the image into red, green, and blue channels. Scroll to see the 3 different channels. The ‘Green’ channel will be used for analysis.

31 Select Image> Adjust > Threshold.

32 Drag the top bar value to 0. Drag the bottom bar value until the entire tissue is red. Press ‘Set’ then ‘Ok’.







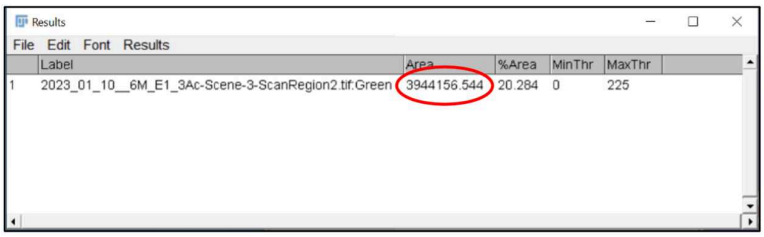
Note

This first threshold may be changed between samples to properly select the total tissue area, ensuring that most of each tissue section appears red.

33 Select Analyze> Set Measurements.

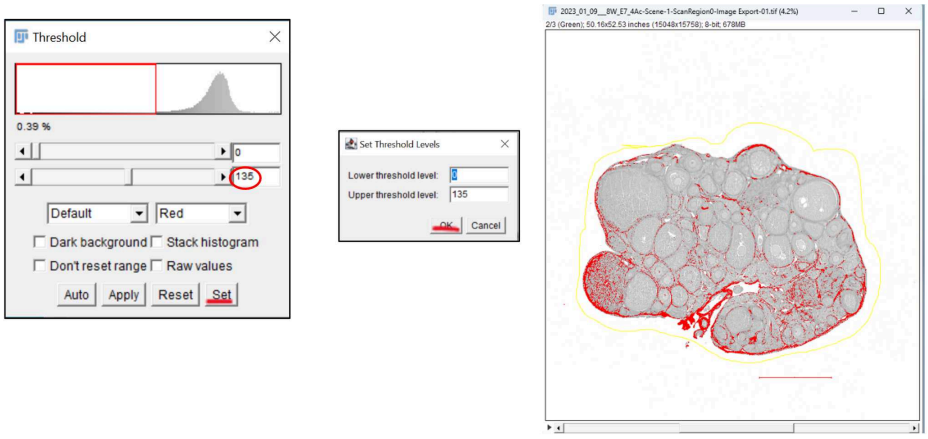
34 Check ‘Area’, ‘Area Fraction’, ‘Limit to Threshold’, and ‘Display Label’. Press Ok.

35 Select Analyze> Measure.



Record the ‘Area’ measurement in the spreadsheet. This output value is the entire area of the tissue.

36 Go back to the threshold window, move the bottom bar until only the positive signal is in red (It can be helpful to have the original image up next to the threshold image to ensure positive signal is being captured correctly).

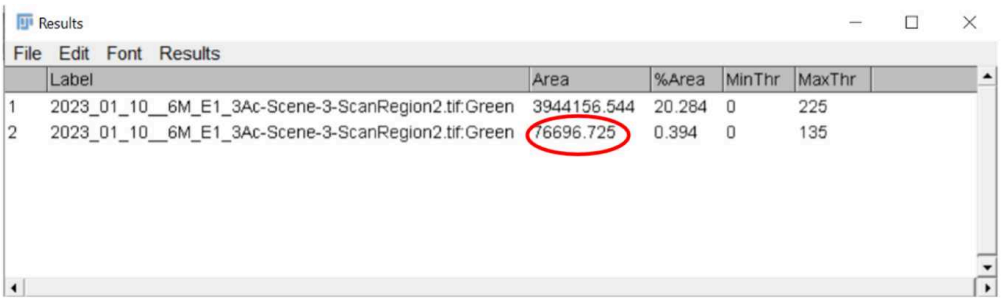


Press ‘Set’ then ‘Ok’. The positive threshold value should be determined using the staining from the oldest animal and applied consistently across all sections in the dataset.

Note

When comparing samples, it is essential to use the same threshold for what is considered positive signal (i.e. the second threshold).

37 Select Analyze> Measure.



Record the ‘Area’ measurement in the spreadsheet. This output is the PSR positive area of the tissue.

38 Divide the positive area measurement by the total area measurement then multiply by to calculate the % of the tissue that is positive for PSR staining.

Protocol references

1. Junqueira, L.C., W. Cossermelli, and R. Brentani, *Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy.* Arch Histol Jpn, 1978. **41**(3): p. 267-74.
2. Junqueira, L.C., G. Bignolas, and R.R. Brentani, *Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections.* Histochem J, 1979. **11**(4): p. 447-55.
3. Briley, S.M., et al., *Reproductive age-associated fibrosis in the stroma of the mammalian ovary.* Reproduction, 2016. **152**(3): p. 245-260.