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# Masson's Trichrome Staining Protocol for FFPE spheroids and microtissues

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The following protocol has been optimized for image analysis-based quantification of collagen within FFPE spheroid samples. It is run on a Leica ST5020 automated stainer. This protocol can be adapted to manual staining.

## PURPOSE:

Trichrome stains are used to differentiate collagen from other tissues. They are usually nuclear and cytoplasmic stains, followed by differentiation, then followed by a collagen fiber stain. Trichrome stains are useful for indicating fibrotic changes.

## PRINCIPLE:

The staining reaction is based on the differential effect of acid dye on collagen and other tissue components.

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spheroid, microtissue, collagen, trichrome, FFPE, staining, quantitative, image analysis, autostainer, histology, Leica ST5020

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1. Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded. If blue staining of connective tissue appears faded, the section has probably been overdifferentiated in the acetic acid solution.
2. Sections fixed in 10% neutral buffered formalin will stain poorly and unevenly if not mordanted in either Bouin or a mercuric chloride solution; however, mercuric fixatives should not be used because of the toxicity.

#### MATERIALS

☒ Acetic acid Glacial Fisher

Scientific Catalog #A38-212

☒ Pro-Par Clearant Anatech

LTP Catalog #510

☒ Trichrome Stain (Masson) Kit Sigma

Aldrich Catalog #HT15-1KT

☒ Isopropyl Alcohol 99% Pacific Southwest Lab

Equipment Catalog #104B

☒ Bouins Fixative Medical Chemical

Corporation Catalog #456-A-2.5GL

#### Solutions and Reagents:

1. Bouin's Fixative (HT15-1KT)
2. Aniline Blue Solution (HT15-1KT)
3. Biebrich Scarlet-Acid Fuchsin Solution (HT15-1KT)
4. Phosphomolybdic Acid Solution (HT15-1KT)
5. Phosphotungstic Acid Solution (HT15-1KT)
6. 1% Acetic Acid Solution
7. Isopropyl Alcohol, 99%
8. Pro-Par Clearant

*Prepare Working Phosphotungstic/Phosphomolybdic Acid (PTA/PMA) Solution by mixing 1 volume of Phosphotungstic Acid Solution and 1 volume Phosphomolybdic Acid Solution with 2 volumes of deionized water.*

*Biebrich Scarlet-Acid Fuchsin and Aniline Blue solutions are ready to use.*

*Prepare a 1% acetic acid solution by adding 4ml of glacial acetic acid to 396ml of deionized water.*

ST5020

Automated Multistainer

Leica

Leica ST5020



Bouin's Fixative contains picric acid. Picric acid containing less than 10% water is very explosive; therefore, it is important that solutions not be spilled in the oven and then allowed to evaporate. For this reason, the staining jar containing Bouin's Fixative/picric acid should be placed inside a secondary container while in the oven.

1. Cut paraffin sections at 4 $\mu$ m.
2. Slides must be completely dry before deparaffinization.
3. Bouin's solution must be preheated to insure proper mordanting.

## Deparaffinization Protocol

31m 30s

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Step	Reagent	Duration	Exact	Dip
1	Oven station	5:00	N	N
2	Oven station	5:00	N	N
3	Pro-par	5:00	N	Y
4	Pro-par	5:00	N	Y
5	Pro-par	5:00	N	Y
6	IPA 99%	1:30	N	Y
7	IPA 99%	1:30	N	Y
8	IPA 90%	1:30	N	Y
9	DI Water station	2:00	N	N

## Mordanting

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  1. For formalin fixed tissue, post-fix deparaffinized slides in Bouin's solution for 1 hour at 56°C or at room temperature overnight. Do not leave in oven overnight. Bouin's solution must be preheated to insure proper mordanting.
  2. Wash in water and discard first two changes to red container aqueous waste. Rinse in sink with running tap water for 5 - 10 minutes to remove the yellow color. Use warm water to remove excess yellow color.

3. Rinse in sink with distilled water for 30 seconds.

### Masson Trichrome Protocol MT3B for Leica ST5020 Autostainer

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Step	Reagent	Duration	Exact	Dip
1	Biebrich Scarlett	5:00	Y	Y
2	DI Water station	3:00	Y	N
3	PTA/PMA Working Solution	5:00	Y	Y
4	Aniline Blue	5:00	Y	N
5	DI Water station	2:00	Y	N
6	Acetic Acid, 1%	2:00	Y	Y
7	IPA, 99%	0:20	Y	Y
8	IPA, 99%	0:20	Y	Y
9	IPA, 99%	1:00	Y	Y
10	IPA, 99%	1:00	Y	Y
11	IPA, 99%	1:00	Y	Y
12	50:50 Pro-par/IPA99	1:00	Y	Y
13	Pro-Par	3:00	N	Y
14	Pro-Par	3:00	N	Y
15	Pro-Par			

### Mounting

4 1. Use a resinous mounting medium to coverslip slides. Allow to dry flat for a minimum of 2 hours.