Campbell Lab

**Staining protocol for Frozen Sections:**

1. Preheat **Bouin’s** Fluid in a water bath to 60°C in a fume hood or very well-ventilated area
2. Fix frozen sections in 10% **formalin** (not Zinc) for 30 minutes
3. Hydrate in distilled water for 3 minutes.
4. Dip in fresh distilled water for 1 minute.
5. Place slide in preheated **Bouin’s** Fluid for 60 minutes
   * Let cool for 5-10 minutes
6. Rinse slide in tap water for 5 minutes to remove the picric acid (*until section is a very light yellow to clear.*)
7. Rinse once in distilled water for 1 minute.
8. Mix equal parts (25mL each if using a Coplin jar) of **Weigert’s** (A) and **Weigert’s** (B) and stain slide with working Weigert’s Iron Hematoxylin for 5-7 minutes. (*Discard after use.)*
9. Rinse slide in running tap water for 2 minutes.
10. Apply **Biebrich Scarlet/Acid Fuchsin** Solution to slide for 5-15 minutes. (*Discard after use.)*
11. Rinse slide in distilled water for 1 minute.
12. Place slides in **Phosphomolybdic/Phosphotungstic** Acid Solution for 10-15 minutes or until collagen is no longer red. *(Discard after use.)*
13. Without rinsing, apply **Aniline Blue** Solution to slide for 5-10 minutes. *(Discard after use.)*
14. Rinse slide in distilled water for 1 minute.
15. Dunk in fresh **Acetic Acid** Solution (1%) for 1 minute.
16. Dehydrate very quickly in 2 changes of 95% **Alcohol** (dipping in), followed by 2 changes of

**100% Alcohol** (2 minutes per change).

1. Clear in X**ylene** (10-15 minutes).
2. Air dry completely.
3. Dip again in Xylene for 10-20 seconds and mount cover slip with DPX.

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| *Step (stain/wash)* | *Time (minutes)* |
| **10% formalin** | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| **Bouin’s** | **60 mins in hot water bath + 5-10 mins cooling at room temp** |
| wash 2 | Rinse for 5 mins in running tap water. Then once with distilled water |
| **Weigert’s** | **5-7 mins** |
| wash 3 | 2 mins running tap water |
| **BS/AF** | **10-15 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| **P/P** | **5-15 mins** |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain |
| **Aniline Blue** | **5-10 mins** |
| wash 5 | Rinse in distilled water |
| **Acetic Acid** | **3-5 mins** |
| final wash & dehydrate | Rinse in distilled water; 2 dips in 95% ethanol, 2 x 2 mins in Absolute Alcohol |

“By use of the three stains, Masson’s Trichrome staining technique is used for the detection of collagen fibers in tissues such as the skin, heart, muscles. The samples are formalin-fixed, paraffin-embedded sections, or frozen sections. Weigert’s Hematoxylin, an iron hematoxylin dye, is used to stain the nuclei. This dye is resistant to decolorization by acidic staining solutions. Biebrich scarlet-acid fuschin solution stains all the acidic tissues such as the cytoplasm, muscle, and collagen. Phosphomolybdic or phosphotungstic acid is used as a decolorizing agent, making the Biebrich Scarlet-acid fuschin diffuse out of the collagen fibers, leaving the muscle cells stained red. Aniline blue stains the collagen along with 1% acetic acid, which is added to show a difference in the tissue sections. The collagen fibers stain blue and the nuclei stains black, with a red background.”

<https://microbenotes.com/massons-trichrome-staining/>

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5-10 mins cooling at room temp** |
| wash 2 | Rinse for 5 mins in running tap water. Then once with distilled water |
| Weigert’s | **~~5 mins~~ 7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **~~10 mins~~ 15 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **~~10 mins~~ 7 & 10 mins** |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain |
| Aniline Blue | **10 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **3-5 mins ~~(both slides 3 mins)~~** |
| final wash & dehydrate | 2 dumps in 95% ethanol, 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5-10 mins cooling at room temp** |
| wash 2 | Rinse for 5 mins in running tap water. Then once with distilled water |
| Weigert’s | **5 mins 7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **10 mins 15 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **10 mins 7 & 10 mins 5 mins** |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain |
| Aniline Blue | **10 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **3 mins [Slide A = 3 mins, Slide B = 5 mins]** |
| final wash & dehydrate | Rinse in distilled water; 2 dips in 95% ethanol, 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* | |
| 10% formalin | **30 mins** | |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water | |
| Bouin’s | **60 mins in hot water bath + 5-10 mins cooling at room temp** | |
| wash 2 | Rinse for 5 mins in running tap water. Then once with distilled water | |
| Weigert’s | **Slide D = 7 mins** | **Slide F = 10 mins** |
| wash 3 | 2 mins running tap water | |
| BS/AF | **Slide D = 20 mins** | **Slide F = 15 mins** |
| wash 4 | Rinse in distilled water for 1 min | |
| P/P | **5 mins** | |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain | |
| Aniline Blue | **10 mins** | |
| wash 5 | Rinse in distilled water | |
| Acetic Acid | **3 mins** | |
| final wash & dehydrate | Rinse in distilled water; 2 dips in 95% ethanol, 2 x 2 mins in Absolute Alcohol | |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Rinse for 5 mins in running tap water. Then once with distilled water |
| Weigert’s | **7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **15 mins; 20 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **5 mins** |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain |
| Aniline Blue | **10 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **3 mins** |
| final wash & dehydrate | 2 dumps in 95% ethanol, 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Rinse for 10 mins in running tap water. Then once with distilled water.   * Rinse Slide G for 5 mins, check under microscope, then rinse for another 5 mins and check for any differences * Rinse Slide N for 10 mins, check under microscope & compare with Slide G |
| Weigert’s | **7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **15 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **5 mins** |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain |
| Aniline Blue | **10 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **Slide G = 3 mins; Slide N = 5 mins** |
| final wash & dehydrate | 1. Rinse in distilled water 2. Dehydrate in graded alcohol (50%  85%) 3. 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse Slide X for 7 mins. * Rinse Slide Y for 10 mins * check under microscope & compare; note any observations |
| Weigert’s | **7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **15 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **5 mins** |
| NO WASH | Do not wash in between these steps; tap-off slide and apply next stain |
| Aniline Blue | **10 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **5 mins? maybe 4 mins?** |
| final wash & dehydrate | 1. Rinse in distilled water 2. Dehydrate in graded alcohol (50%  85%) 3. 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Rinse slides under running tap water until yellow is completely removed |
| Weigert’s | **7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **Slide *X* = 15 mins; Slide *Y* = 20 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **10 mins** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **5 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **4 mins** |
| final wash & dehydrate | 1. Rinse in distilled water 2. Dehydrate in graded alcohol (50%  85%) 3. 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 3 x 3 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Rinse slides under running tap water until yellow is completely removed |
| Weigert’s | **7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **20 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **10 mins** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **5 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **Slide *K* = quick dunk; Slide *L* = 2 min** |
| final wash & dehydrate | 1. Rinse in distilled water 2. Dehydrate in graded alcohol (50%  85%) 3. 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins; Slide *P* = NO FORMALIN** |
| wash 1 | 9 mins running tap water + 3 mins hydrating in distilled water |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Slide *RQ* = 5 minute wash  Rinse slides under running tap water until yellow is completely removed |
| Weigert’s | **7 mins** |
| wash 3 | 2 mins running tap water |
| BS/AF | **Slide *RQ* = 15 mins; Slide *O* = 30 mins; Slide *P* = 20 mins** |
| wash 4 | Rinse in distilled water for 1 min |
| P/P | **5 mins** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **10 mins** |
| wash 5 | Rinse in distilled water |
| Acetic Acid | **3 mins** |
| final wash & dehydrate | 1. Rinse in distilled water 2. Dehydrate in graded alcohol (50%  85%) 3. 2 x 2 mins in Absolute Alcohol |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 9 mins running tap water  3 mins hydrating in D.I. H2O |
| Bouin’s | **75 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Rinse in running tap water *until all yellow is removed*  3 mins hydrating in D.I. H2O |
| Weigert’s | **20 mins** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **20 mins** |
| wash 4 | Rinse in running tap water until runoff is light-pink to clear |
| P/P | **10 mins (periodically check for evidence of red counterstaining)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **15-20 mins [Slide Q = 10 mins; Slide 1F = 20 mins]** |
| wash 5 | *Do not wash with H2O*; briefly rinse off by pipetting 1% Acetic Acid onto slide to get most of the blue off before next step |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | 1. Briefly rinse off with D.I. H2O 2. Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each 3. Let dry before viewing under microscope |

* ***NOTE:*** do not dry so thoroughly with Kimwipes; just flick off as much as possible and move on

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | 9 mins running tap water  3 mins hydrating in D.I. H2O |
| Bouin’s | **75 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | Rinse in running tap water *until all yellow is removed*  3 mins hydrating in D.I. H2O |
| Weigert’s | **7 vs. 14 mins [Slide \_\_\_ = 7 mins; Slide \_\_\_ = 14 mins]** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **20 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10 mins (periodically check for evidence of red counterstaining)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off by pipetting 1% Acetic Acid onto slide to get most of the blue off before next step |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | 1. Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each 2. Let dry before viewing under microscope |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60-65 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **5 vs. 10 mins [Slide \_\_\_ = 5 mins; Slide \_\_\_ = 10 mins]** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **20 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-15 mins (monitor for evidence of red counterstaining)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off blue stain by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60-65 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **8 mins** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **20 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-20 mins [Slide S = 10 mins; Slide T = 20 mins]** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off blue stain by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **[Slide U = 3 mins; Slide W = 6 mins]** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **25 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-15 mins (monitor for red counterstaining)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off blue stain by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **3 mins** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **25 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-20 mins (monitor for red counterstaining)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off blue stain by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **Slide 1K = 3 mins as usual; Slide 1L = 3 mins facedown** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **25 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-20 mins (monitor for red counterstaining)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off blue stain by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |

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| --- | --- |
| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60 mins in hot water bath + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **3 mins inverted (face down) in petri dish** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **25 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-20 mins (*monitor for red counterstaining*)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off A.B. by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |

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| *Step (stain/wash)* | *Time (minutes)* |
| 10% formalin | **30 mins** |
| wash 1 | * 9 mins running tap water * 3 mins hydrating in D.I. H2O |
| Bouin’s | **60 mins @ 60°C + 5 mins cooling at room temp** |
| wash 2 | * Rinse in running tap water *until all yellow is removed* * 3 mins hydrating in D.I. H2O |
| Weigert’s | **3 mins inverted (face down) in petri dish; cover with top of petri dish** |
| wash 3 | Rinse in running tap water for 10 mins |
| BS/AF | **22 mins** |
| wash 4 | Rinse in running tap water until runoff is light pink to clear |
| P/P | **10-20 mins; check at 10 and 15 mins (*monitor for red counterstaining*)** |
| NO WASH | *Do not wash in between these steps*; tap-off slide and apply next stain |
| Aniline Blue | **20 mins** |
| wash 5 | *Do not wash with H2O*; briefly rinse off A.B. by pipetting 1% Acetic Acid onto slide |
| 1% Acetic Acid | **2 x quick dunks in 1% Acetic Acid** |
| final wash & dehydrate | * Dehydrate in graded alcohol (75%  90%  100%) for 3 secs each * Let dry before viewing under microscope |