Haematoxylin staining methods

WOLF D. KUHLMANN

Laboratory Diagnostics & Cell Science, 56112 Lahnstein

Subsequent to immunohistological procedures, counterstaining of tissue sections is helpful for the study of histological and histopathological details. Generally, counterstains are used to obtain supplementary information to that of the primary stain. An effective counterstain should not intrude the major stain, i.e. the immunostained antigen in the tissue section.

The right selection of a counterstain will result in important additional information. In most cases, the overall morphology of the tissue section or the staining of nuclear morphology is desired. For the latter, a variety of haematoxylin formulations exist. With our DAB reactions we obtain nice conterstains by using GILL'S or MAYER'S haematoxylins.

Haematoxylin is obtained from the logwood *Haematoxylum campechianum*, a tree indigenous to Central America by several extraction and precipitation methods. Haematoxylin can be also produced synthetically, but the natural product is still the agent in common use. The dye was originally used for industrial purpose. In the first attempts to use haematoxylin as a direct dye for histological specimens only poor results were obtained.

Campeche: The bluewood tree or campeche tree (Haematoxylum campechianum) is a plant species found in Central America and the northern part of South America that belongs to the carob subfamily (Caesalpinioideae). Bluewood can be used to dye wool, cotton, linen and silk. The substance of bluewood used in histological techniques is haematoxylin. There are over 100 different recipes for the production of colour solutions containing haematoxylin.

The potential for haematoxylin in microscopy has been first realized by F BÖHMER (1865) who combined a metallic mordant with haematoxylin to stain tissue sections adequately.

Since then, haematoxylin dye solutions contain haematoxylin and an alum (hemalum, alum haematoxylin). Coupling of haematoxylin with a mordant is still the major form in which this dye is used. Many formulations have been devised in the meantime. They vary in the amount of haematoxylin, the type and quantity of aluminium salts, solvents, oxidizing agents and stabilizers. Also, other ingredients may be added. They are not essential but can modify the behaviour of the haematoxylin dyes.*

Formulations of haematoxylin stains

Since the introduction of haematoxylin as histological dye, many staining formulations have been suggested. Here, examples of the enduring haematoxylin staining methods are given.

Haematoxylin (Delafield)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Ammonium aluminium sulfate (ammonium alum) Sodium iodate Glycerol Sodium hydrogen carbonate Ethanol Distilled water	• Ammonium alum stock solution: saturated ammonium alum in distilled water (about 10%) • Haematoxylin stock solution: 1.0 g haematoxylin dissolved in 50.0 mL 96% ethanol • Sodium iodate stock: 10.0 g sodium iodate dissolved in 100.0 mL distilled water • DELAFIELD stock solution: 50.0 mL haematoxylin stock *plus* 2.0 mL sodium iodate stock mix and wait for 10 min, add 160 mL ammonium alum stock mix vigorously for 1 min, add 40.0 mL glycerol the final mixture is filtered • DELAFIELD dye solution: Delafield haemalum stock solution 1:40 diluted with distilled water • 0.1% sodium hydrogen carbonate in distilled water
Staining procedure Immuno-stained sections are passed through distilled water and stained: – DELAFIELD dye solution 1-6 hours – running tap water 30 min – sodium hydrogen carbonate under microscopic control	

* Dyes and other chemicals in histological staining can be toxic. They must be handled with care

(to ‘blue’)	
– distilled water	2 x 1 min
Slides are dehydrated in ascending series of ethanol and mounted under coverglass	

Haematoxylin (Ehrlich)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Potassium aluminium sulfate (potassium alum) Potassium iodate Isopropanol Glycerol Glacial acetic acid HCl (25%) Sodium hydrogen carbonate Ammonia aqueous Distilled water	• EHRLICH haemalum dye solution: 1.0 g haematoxylin dissolved in 50.0 mL 96% isopropanol the following substances are added in the order given: (a) 50.0 mL distilled water (b) 50.0 mL glycerol (c) 1.5 g potassium alum (d) 5.0 mL glacial acetic acid mix, add 0.2 g potassium iodate and mix again the dye solution is ready for use • 0.1% HCl solution in distilled water • 0.1% sodium hydrogen carbonate in distilled water • Ammonia water: 50 µL ammonia aqueous in 100 mL distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:

– EHRLICH haemalum dye solution	2-15 min
– running tap water	30 min
– sodium hydrogen carbonate (to ‘blue’)	under microscopic control
– differentiate in HCl solution (when color is too strong)	under microscopic control
– rinse in tap water	1 min
– ammonia water (to ‘blue’)	under microscopic control
– running tap water	30 min
– distilled water	2 x 1 min

Slides are dehydrated in ascending series of ethanol and mounted under coverglass

Haematoxylin (Gill)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Aluminium sulfate Ethylene glycol Sodium iodate Glacial acetic acid HCl (25%) Sodium hydrogen carbonate Ammonia aqueous Distilled water	• GILL haematoxylin dye solution (triple): 6.0 g haematoxylin dissolved in 750.0 mL distilled water *plus* 250.0 mL ethylene glycol *plus* 0.6 g sodium iodate *plus* 80.0 g aluminium sulfate *plus* 20 mL glacial acetic acid Reagents are combined in the order given, mix for 1 hour at room temperature; the dye solution is ready for use • 0.1% HCl solution in distilled water • 0.1% sodium hydrogen carbonate in distilled water • Ammonia water: 50 µL ammonia aqueous in 100 mL distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:

– GILL haematoxylin dye solution (triple)	2-15 min
– running tap water	30min
– sodium hydrogen carbonate (to 'blue')	under microscopic control
– differentiate in HCl solution (when color is too strong)	under microscopic control
– rinse in tap water	1 min
– ammonia water (to 'blue')	under microscopic control
– running tap water	30 min
– distilled water	2 x 1 min

Slides are dehydrated in ascending series of ethanol and mounted under coverglass

Haematoxylin (Hansen)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Ammonium ferric sulfate (iron alum) Ethanol	• Iron alum stock solution: 4.5 g iron alum dissolved in 65.0 mL distilled water • Haematoxylin stock solution:

HCl (25%) Sodium hydrogen carbonate Distilled water	0.75 g haematoxylin dissolved in 35.0 mL distilled water • HANSEN haematoxylin dye solution: iron alum stock and haematoxylin stock solutions are mixed and heated until the boiling point is reached let the final mixture cool down and filter • HCl-ethanol solution: 70.0 mL absolute ethanol *plus* 26.0 mL distilled water *plus* 4.0 mL HCl (25%) • 0.1% sodium hydrogen carbonate in distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:

– HANSEN haematoxylin dye solution	30 min
– running tap water	1-5 min
– differentiate in HCl-ethanol solution (when color is too strong)	under microscopic control
– rinse in tap water	1 min
– sodium hydrogen carbonate (to 'blue')	under microscopic control
– running tap water	30 min
– distilled water	2 x 1 min

Slides are dehydrated in ascending series of ethanol and mounted under coverglass

Haematoxylin (Harris)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Potassium aluminium sulfate (potassium alum) Ethanol Mercury (II) oxide (mercuric oxide) Glacial acetic acid HCl (25%) Sodium hydrogen carbonate Ammonia aqueous Distilled water	• Potassium alum stock solution: 20.0 g potassium alum dissolved in 200.0 mL distilled water (using heat if necessary) • Haematoxylin stock solution: 1.0 g haematoxylin dissolved in 10.0 mL absolute ethanol • HARRIS haematoxylin dye solution: haematoxylin stock and potassium alum stock solutions are mixed bring rapidly to the boil and carefully add 0.5 g mercuric oxide (little at a time because of foaming!);

	cool the mixture rapidly by immersing the flask into iced water the dye solution is ready to use as soon as it is cool the stain is filtered prior to use optinonally: addition of 1.0 mL glacial acetic acid (for sharper nuclear staining) • 0.1% HCl solution in distilled water • 0.1% sodium hydrogen carbonate in distilled water • Ammonia water: 50 µL ammonia solution in 100 mL distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:

–	HARRIS haematoxylin dye solution	1-5min
–	running tap water	10 min
–	sodium hydrogen carbonate (to 'blue')	under microscopic control
–	differentiate in HCl solution (when color is too strong)	under microscopic control
–	rinse in tap water	1 min
–	ammonia water (to 'blue')	under microscopic control
–	running tap water	30 min
–	distilled water	2 x 1 min

Slides are dehydrated in ascending series of ethanol and mounted under coverglass

Haematoxylin (Heidenhain)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Ammonium ferric sulfate (iron alum) Ethanol Sodium iodate Glacial acetic acid Sulfuric acid Sodium hydrogen carbonate Distilled water	• Haematoxylin stock solution: 1.0 g haematoxylin dissolved in 20.0 mL 96% ethanol *plus* 180.0 mL distilled water *plus* 0.2 g sodium iodate (solution is ready to use) • HEIDENHAIN haematoxylin dye solution: 50.0 mL haematoxylin stock dissolved in 50.0 mL distilled water • Iron alum solution (I): 4.0 g iron alum dissolved in

	96.0 mL distilled water and the following substances are added: (a) 1.0 mL glacial acetic acid (b) 1.2 mL sulfuric acid • Iron alum solution (II): 2.5 g iron alum dissolved in 97.5 mL distilled water • 0.1% sodium hydrogen carbonate in distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:

– Iron alum solution (I)	1-6 hours
– HEIDENHAIN haematoxylin dye solution	1-3 hours
– distilled water	rinse
– differentiate in iron alum solution (II)	under microscopic control
– running tap water	10 min
– sodium hydrogen carbonate (to 'blue')	under microscopic control
– running tap water	30 min
– distilled water	2 x 1 min

Slides are dehydrated in ascending series of ethanol and mounted under coverglass

Haematoxylin (Mayer)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Potassium aluminium sulfate (potassium alum) Sodium iodate Chloralhydrate Citric acid HCl (25%) Sodium hydrogen carbonate Ammonia aqueous Distilled water	• Haematoxylin stock solution: 1.0 g haematoxylin dissolved in 1000 mL distilled water *plus* 0.2 g sodium iodate *plus* 50.0 g potassium alum • MAYER haematoxylin dye solution: 100.0 mL haematoxylin stock *plus* 5.0 g chloralhydrate *plus* 0.1 g citric acid solution is mixed and filtered prior to use • 0.1% HCl solution in distilled water • 0.1% sodium hydrogen carbonate in distilled water • Ammonia water: 50 µL ammonia solution in 100 mL distilled water

Staining procedure

Immuno-stained sections are passed through distilled water and stained:	
– MAYER haematoxylin dye solution	5-10 min (microscopic control)
– running tap water	30 min
– sodium hydrogen carbonate (to 'blue')	under microscopic control
– differentiate in HCl solution (when color is too strong)	under microscopic control
– rinse in tap water	1 min
– ammonia water (to 'blue')	under microscopic control
– running tap water	30 min
– distilled water	2 x 1 min
Slides are dehydrated in ascending series of ethanol and mounted under coverglass	

Haematoxylin (Weigert)

Chemicals	**Chemical solution**
Haematoxylin (C.I. 75290) Iron (III) chloride (ferric chloride) Ethanol HCl (25%) Sodium hydrogen carbonate Distilled water	• Haematoxylin stock solution: 1.0 g haematoxylin dissolved in 100.0 mL 96% ethanol mixture should mature for 1 week • Iron (III) chloride stock solution: 1.16 g iron (III) chloride dissolved in 99.0 mL distilled water *plus* 1.0 mL HCl (25%) • WEIGERT haematoxylin dye solution: 100 mL haematoxylin stock *plus* 100 mL iron (III) chloride stock are mixed prior to use • 0.1% sodium hydrogen carbonate in distilled water

Staining procedure	
Immuno-stained sections are passed through distilled water and stained:	
– WEIGERT haematoxylin dye solution	1-5 min
– running tap water	30 min
– sodium hydrogen carbonate (to 'blue')	under microscopic control
– running tap water	30 min
– distilled water	2 x 1 min
Slides are dehydrated in ascending series of ethanol and mounted under coverglass	

For more informations of mordants and mordanted haematoxylins for histological staining see BD LLEWELLYN's Stains File (http://www.stainsfile.info/StainsFile/stain/hemindex.htm).

Progressive and regressive staining

Haemalum solutions (alum haematoxylin) can be used as *progressive* or *regressive* stains. In the latter case, tissue sections are at first overstained, and some of the dyeing material must be removed. The quantity to be removed depends on both the particular dye formula and the personel preference of the microscopist; there is no correct degree. For the purpose of immunohistology, a clean background with clear and sharp nuclear staining is preferred. Very clean nuclei staining are obtained by treatment of haematoxylin stained sections with 0.1-1.0% alum solutions. Alum solutions are also useful to correct overstainings.

Excess stain is removed by procedures which are called *differentiation* which is done in several ways. Very common are extraction by acids and by mordants. The former method uses diluted acid (e.g. 0.1-1.0% HCl in water or in ethanol), while the latter relies on a mordant at reduced strength (f.e. in the iron haematoxylin staining method of Heidenhain).

In order to improve control over the differentiation, the acid solution is prepared in 70% ethanol (f.e. 0.1% HCl in 70% ethanol). The section will get reddish in the acid solution, and the process should be controlled microscopically. Thereafter, sections must be washed carefully followed by 'bluing'. 'Bluing' of the sections is performed either in running tap water or with diluted sodium hydrogen carbonate or with ammonia water.

References for further readings

Böhmer F (1865)
Heidenhain M (1885)
Ehrlich P (1886)
Mayer P (1891)
Mayer P (1892)
Heidenhain M (1894)
Mayer P (1896)
Harris HR (1900)
Weigert K (1904)
Weigert K (1928)
Baker JR and Jordan BM (1953)
Baker JR (1962)
Romeis B (1968)
Lillie RD and Pizzolato P (1972)
Gill GW *et al*. (1974)
Clark G (1974)
Lillie RD *et al*. (1974)
Llewellyn BD (1974)
Lillie RD *et al*. (1975)
Lillie RD and Fullmer HM (1976)
Bracegirdle B (1978)
Kuhlmann WD (1978)
Kuhlmann WD and Wurster K (1980)

Stevens A (1990)
Bettinger CL and Zimmermann HW (1991a, 1991b)
Horobin RW and Kiernan AJ (Conn's Biological Stains, 2002)

Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

 06.10.2023