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A Discussion of Multicolor Stains

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INTRODUCTION

Staining is a very necessary part of histological procedure for without stain a cleared section of tissue is almost invisible and certainly there is little differentiation of cell types and cell contents possible. Practically all staining is done with either Delafield's or Heidenhain's hematoxylin. Heidenhain's hematoxylin is usually used for precise cytological work while Delafield's is used for the study of tissues. Delafield's stains nuclear and other electronegative structures deep blue. Eosin is usually used as a contrast stain while Heidenhain's hematoxylin stains electornegative material black and is usually used without a counterstain.

There are many types of special stains which are valuable in demonstrating specific features such as the Golgi-Cajal method of staining for neurological study or the Romanowsky stains for the differentiation of blood cells.

A third type of stain is the multicolored stains. These stain each type of tissue or cell inclusion a different color. With the common stains a tissue must be identified by its histological characteristics; with multicolor stains, the tissue is identified by its staining reaction. The various parts of a cell may also be identified by color rather than shape or position. This is the type of stain with which this paper deals.

Most of the multicolored stains have been developed by one person, used by several and then relegated to the position in literature of "Dr. Doe states that such and such is also a successful stain". The basic reason for this is that no two batches of stain are of the same chemical composition. These multicolored stains require the concentrations to be controlled very accurately and these concentrations many be arrived at only by trial and error because of the variability of the dyestuffs. Because of this, it takes almost as much time to develop a staining solution from directions as it does to work out a new stain.

The most common of the multicolored stains is Mallory's triple connective tissue stain. The three dyes used are; acid fuchsin, water soluble aniline blue, and orange G.

Acid fuchsin is derived from basic fuchsin by sulphonation There are four possible basic fuchsins and each of them may yield a mon-, a di-, or a tri-sulphonic acid. This causes the manufacture of a uniform type to be very difficult if not impossible.

Acid fuchsin is a widely used cytoplasmic stain but finds even greater use in special procedures such as the Van Gieson stain, the Ehrlich Biondi Heidenhain stain, the Pianese cancer stain, and the Mallory connective tissue stain. It stains cytoplasm, nuclear spindles, asters, and

particularly brings out connective tissue. It is highly soluble in water and is easily removed by alkalies. Acids strengthen the stain and give sharper definition.

Aniline blue is also an acid dye formed by sulphonation. The water soluble form is formed by sulphonation of triphenyl rosanilin hydrochloride. It is always a mixture of even more unpredictable composition than acid fuchsin. Water soluble aniline blue is used under so many different names that it is difficult to find much about its properties. Lee says that it gives very good differentiation of nerve tissue and of cartilage. This ability to stain cartilage is used in the Mallory technique.

Orange G, another acid dye, is the benzazo beta naphthol disulphonate of soda. It is one of the most important counterstains and the orange stain most frequently used by histologists.

Flemming's triple stain, employing safranin, gentian violet, and orange G, is used for exact cytological work. It is claimed to stain chromosomes bright red, cytoplasm yellow, and the spindles blue or violet.

Safranin, which is a basic stain of the azin group, is formed by the oxidation at high temperatures of a mixture of paradiamines and primary amines or phenols. Of the many types of safranin, the one most desirable is a mixture of dimethyl

and trimethyl phenosafranins having a light absorption curve with a maximum of 515 mu. The German firm of Grubler Company manufacture a type called safranin O which is very satisfactory but which is unobtainable at this time. Safranin is used to stain chromatin in the Flemming procedure. Plant histologists use it to stain lignified and cutinized tissues and proteins.

According to the definition of the Commission on the Standardization of Biological Stains, gentian violet is either pentamethyl or hexamethyl pararosanilin or it may be a mixture composed primarily of these two. Two grades are recognized; gentian violet bluish, which is apparently crystal violet;and gentian violet reddish, which is probably a mixture of one of the deeper methyl violets. It is soluble in both water and alcohol and has its chief value as a nuclear stain. It has been used recently for its inhibiting action upon bacteria and has been suggested as a therapeutic agent.

The Ehrlich Biondi Heidenhain triple stain uses acid fuchsin, orange G, and Methyl green.

Methyl green, which is the most common aniline dye in commerce, is the methyl chloride of hexa methyl para rosanilin chloride. It is prepared from crystal violet and is almost impossible to completely free from the crystal violet. This at least partially explains the metachromatic action of methyl

green. This stain is particularly sensitive to alkalies; therefore it must be used in an acidic or absolutely neutral solution. It is an extremely valuable stain for nuclear or chromatin material and is used for fresh unfixed tissues. Methyl green is a progressive stain. A solution of methyl green is very penetrating and kills cells instantly without changing their form in any way, so that it may be used as an exceedingly delicate fixative. Tissues stained with methyl green are difficult to mount in balsam because the stain is so soluble in alcohol that it is all dissolved by the time the section is dehydrated. Accordingly, aqueous mounts of tissues stained with this dye are almost always used. Even with aqueous mounts, however, the medium must be charged with color to prevent fading of the tissue.

Another staining procedure requires the use of orcein, azocarmine G, and naphthol green B. This stain is used for connective tissue-muscle differentiation.

Orcein is a dye obtained from the lichen, Lecanora Parella; but is not to be confused with orcin, another derivative of this plant. It is said to unite the properties of both acidic and basic stains, and to be capable of staining the tissue a combination of contrasting colors. Israel stains in a solution containing; two grams of orcein, two grams of glacial acetic acid, and one hundred cubic centimeters of

distilled water. The nuclei stain blue while the cytoplasm is red.

Lee mentions a stain called Bonney's triple stain. The stains used are; methyl violet, pyronin, and orange G. It stains chromatin violet, cytoplasm red, connective tissue yellow and keratin violet.

A stain described in Biological Abstracts uses a triple mordant composed of aluminum chloride, iron chloride, and calcium chloride. Hematoxylin iodate and alizarin red S are the stains used to form lakes with this mordant.

For best results in staining with Mallory's triple connective tissue stain, Lee and Galigher both advise fixing the tissue in Zenker's fluid. This is made up of:

Corrosive sublimate	5.0	grams
Potassium bichromate	2.5	grams
Sodium sulphate	1.0	grams
Distilled water	100.0	cc
Glacial acetic acid	5.0	cc

After immersion in this solution for twelve hours, the tissue, trachea of a rabbit, was placed in running water for eighteen hours. This washed most of the excess fixative from the tissue. It was then placed in thirty five percent alcohol for four hours, fifty percent for four hours and then into seventy five percent alcohol plus Lugol's solution.

Lugol's solution is composed of iodine, potassium iodide, and distilled water. The iodine ions in the solution combine with the mercury compounds that may have been precipitated in the tissue and form soluble mercuric iodide. The tissue was allowed to remain in Lugol's solution with several changes for eight hours, then placed in ninety five percent alcohol, and later in terpinol. It was imbedded in paraffin having a melting point of fifty four to fifty six dgrees centigrade, and cut at twelve mu. A portion of the aorta of a rabbit was also prepared; as this is much thinner and lighter than the trachea, it was not left in the various solutions as long. After mounting the sections on slides, the paraffin was removed by immersion in toluene and the sections were run down the alcohol series to water.

Galigher recommends that the sections be stained for five to ten minutes in a .2 % solution of acid fuchsin, rinsed quickly in water and placed in the following solution.

Water soluble aniline blue	.5	grams
Orange G	2.0	grams
1 % aqueous solution of phosphomolybdic acid	100.0	cc

The sections should be allowed to remain in this stain for ten to twenty minutes and then washed in water and later in ninety five percent alcohol. When the red ceases to flow from them, they should be transfered to absolute alcohol and

differentiated to just the desired degree. Before mounting, the slides should be placed in carbol xylol and two changes of xylol.

The fuchsin solution as [redacted] recommended by Galigher was found to be too strong. A .1 % solution for four minutes was sufficient to color the specimen. The mixture of aniline blue and orange G contained too much blue, so that there was little differentiation possible. By changing the proportion of blue to orange from one to four to one to six, the writer was able to introduce some orange into the stained slide. The phosphomolybdic acid acts as a mordant in this stain. Some stains are differentiated by immersion in a weak solution of the mordant. This does not seem to apply to this case for immersion in .5 % phosphomolybdic acid seemed to cause the stain to become more furry rather than more acute. Toluene was used as a clearing agent rather than carbol xylol and xylol.

Galigher advises fixing in Flemming's, Hermann's, Karpechenko's, or chrome acetic acid fluid for staining with Flemming's triple stain. One submaxillary gland of a rabbit was fixed in Flemming's solution, and the other was fixed in Hermann's fluid. Flemmings fluid is made up of:

Chromic acid	.25 %
Osmic acid	.10 %
Glacial acetic acid	.10 %

Hermann's fluid is:

1 % platinum chloride	15	parts
Glacial acetic acid	1	part
2 % osmic acid	4	parts

The tissues were left in these solutions over night and then washed in running water for twenty four hours. They were then taken up the series of alcohols to seventy five percent. At this point they were bleached according to Mayer's chlorine method. A few drops of hydrochloric acid were placed in a vial and potassium chlorate was added. When the chlorine started to evolve, seventy five percent alcohol was added. The tissues, which were left in this solution for two days, were then dehydrated completely and imbedded. Each of these tissues were then cut at twelve mu. Following Galigher's recommendations, the slides were run down the series to fifty percent alcohol and then placed in a 1 % solution of safranin in fifty percent alcohol. After they remained in this solution for twenty four hours, the excess stain was washed out in fifty percent alcohol until the cytoplasm and the spindles were pink. They were then rinsed in water and placed in a one percent solution of gentian violet for twenty minutes. Galigher says the spindles whould be stained violet at this time but that the chromatin should not. After rinsing in water the slides were placed in one percent orange G solution for

about thirty seconds. Because the violet is washed out by this solution, exposure should be carefully regulated. The excess orange and more of the violet are removed by ninety five percent alcohol and then by absolute alcohol. The slides were then flooded with clove oil and placed under the microscope in order that the extraction of the gentian violet could be observed. When differentiation reached the desired point, the slide was immersed in toluene. After transferring to fresh toluene, the section was covered.

The results with this stain were rather unsatisfactory. All of the stained sections were red and orange but there was no differentiation of parts. The gentian violet stained the whole tissue rather than selectively staining the spindles. Part of the trouble may have been caused by the pH of the tissue. The acid of the Mayer's chlorine solution may have lowered the pH so much that the stains would not react as they should have. The fixatives used may have been of the wrong concentrations. The vapors of osmic acid will cause blindness and so it was weighed rather hurriedly and perhaps slightly inaccurately. As only a small quantity of fixative was made up, this may have thrown the solution off. Platinum chloride is very expensive and is in a lumpy form. To prevent loss of this material, it was not reduced to a powder, and so the weighing of this also may have been inaccurate.

Sections to be stained with the Ehrlich Biondi Heidenhain mixture should be fixed in saline sublimate solution. There are three types of this solution listed by Lee. Gaule's solution is:

Corrosive sublimate	5.	grams
Sodium chloride	.5	grams
Distilled water	100.	cc

Heidenhain's saline sublimate solution consists of a one half percent solution of sodium chloride saturated with corrosive sublimate while hot. Lang's solution is composed of:

Distilled water	100	parts
Sodium chloride	6 -10	parts
Acetic acid	6 - 8	parts
Corrosive sublimate	3 -12	parts

Heidenhain's is the poorest of these solutions because it causes precipitates to form in the tissue which are partially soluble in water. The spleen, the trachea, and the aorta of a rabbit were fixed in Gaule's solution. These tissues were treated with Lugol's solution before they were imbedded.

The staining solution is made up of four parts of saturated solution of acid fuchsin, seven parts of saturated solution of orange G, and eight parts of a saturated solution of methyl green. About twenty grams of acid fuchsin, eight grams of orange G, and eight grams of methyl green will dissolve in one hundred cubic centimeters of water. Before using the

concentrated stock solution, it should be diluted with fifty to one hundred volumes of water. The sections should be stained twenty four hours and then rinsed in water acidulated with acetic acid. This stain is progressive rather than regressive in action and so requires no differentiation. The stained sections should be dehydrated quickly, for the methyl green is very soluble in alcohol. Cutting the sections very thin speeds dehydration. If the sections are soaked in a very weak solution of acetic acid for several hours before staining, the staining is more precise. An excess of acidity will cause the acid fuchsin to become a diffuse stain rather than sharply limited to the plastin elements.

SUMMARY

With the aid of multicolor stains, the advanced histologist or cytologist is able to demonstrate structures that would otherwise be invisible. For normal use, however, the multicolor stains are too difficult to prepare and use. The tissue must be in just the right state of fixation, the pH must be right and other rather unpredictable factors must be in the proper balance to insure optimum staining. The common stains do not have to have this careful preparation of tissues, and so preparation for and staining in them is much more rapid than in the case of multicolor stains. This is a definite advantage in some pathological work. Therefore, it may be concluded that, while there is a definite place for multicolor stains, they will never supercede the common hematoxylin stains for general work.

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