Differential Staining With Acid Dyes

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Differential Acid Dyeing

It is quite possible to stain different tissue components in different colours with different acid dyes. There are a number of such methods of varying usefulness ranging from simple stains to differentiate between muscle and collagen to quite involved techniques for staining fibrin in contrast to erythrocytes, or methods for differentiating the cells of the pituitary. There are also methods that incorporate procedures for elastic fibres and mucin, these being the quad and pentachrome methods.

The two most common differential acid dyeing methods, however, are likely the van Gieson and Masson's trichrome. Van Gieson's method is a simple one-step trichrome for differentiating between muscle and collagen, often used as a counterstain. Masson's trichrome is a more complex multi-step procedure for essentially the same thing.

Like much that is done in histology, there are some default assumptions made with these methods. One is the colour scheme. Usually this is yellow erythrocytes, muscle and cytoplasm with red collagen in the van Gieson type, and yellow erythrocytes, red muscle and blue or green collagen in the case of the Masson type.

With the Masson type at least, there is no real reason why other colour schemes would not work. In fact, Masson himself recommended a variant of his method in which the collagen was coloured by metanil yellow. The traditional colour scheme has become popular due to the good contrast it gives, but it can be changed easily by substituting dyes of the appropriate colour at the appropriate point in the method, with some experimentation of course.

Even the van Gieson is not cast in stone. When first introduced there were a spate of copy cat methods using dyes other than acid fuchsin. Methyl blue was one, but an interesting recommendation was the dye naphthol blue black CS to replace acid fuchsin. This dye colours more than just the collagen and was suggested as a dye method for reticulin. It certainly stains more than collagen.

Differential acid dye staining methods generally fall into three distinct groups. The underlying principles have been extensively discussed by Baker(Baker 1958).

1. ***One-Step*** methods, in which the dyes and other ingredients are combined in a single solution. The different components then stain the tissue differentially.
2. ***Multi-Step*** methods, in which single dye solutions are applied in a series of timed sequential steps, each step being optimised, and often using a polyacid.
3. ***Yellowsolve*** methods, in which a red dye is used to overstain all tissue then is differentiated by a yellow dye applied in a water free low polarity solvent.

In these methods there are two components to consider. The first is the tissue and its components. The second is the dyes that are used.

Fixation

Acid dye staining is not confined to eosin and its substitutes, of course. They form a large part of the staining techniques we use regularly. As with hemalum nuclear staining, proper preparation of the tissue is essential for the most reliable and consistent results. Unfortunately simple formalin fiixation does not bring about the brightest acid dye staining, although it is possible to use it with acceptable results.

Probably the most effective fixation for acid dye staining is mercuric chloride, as formal sublimate or B5. Both of these fixatives preserve tissues such that both acid and basic dye staining is enhanced. Morphological preservation is excellent as well. Unfortunately, mercury in all its forms is now approaching the status of a pariah due to its cumulative toxic effects and its impact on the environment. It is possible to still use it, but it does require that special handling and careful attention be given to ensure none is released into the environement. This necessitates the collection of all fluids used, and precipitation of the mercury salts for recycling in facilities capable of processing heavy metals. This makes using it extremely invonvenient, but not impossible. In some places mercury based fixation has been banned outright, and there is a distinct movement in North America to stop using it and find a replacement. The replacement most often mentioned is formal zinc chloride, or B+. This is OK and better than NBF, but it is not a patch on formal sublimate.

A second recommended fixative is Bouin's picric acid-formalin-acetic mixture. Acid dyes often stain brightly after fixation with Bouin's fluid, but nuclear preservation is less effective as the picric acid may hydrolyse the DNA. Once again, however, there is a move to reduce the use of picric acid. It is also

known as trinitrophenol, and is closely related to trinitritoluene or TNT, and it and its salts are explosive in the dry state. In fact, the biggest explosion in Canada occurred when a ship carrying munitions exploded in Halifax Harbour, Nova Scotia, involving picrates which were part of the cargo.

Although at one time it was a standard explosive, its danger is somewhat overstated. In laboratories we always keep it damp or wet with either water or ethanol, and good laboratory practices should ensure that lids and containers are kept clean and free from dried chemicals or crystals formed from wicking.. Under these conditions it does not explode and is quite safe. Providing all solutions are made by converting dry weights of picric acid to equivalent volumes of saturated aqueous or ethanolic solutions, it can be used with complete safety.

Bouin's fluid is valuable also as a fixative that can be used to refix sections after they have been cut and picked up on the slide. Placing a dewaxed and hydrated section into Bouin's fluid overnight or in an incubator at 65°C for an hour can noticeably alter the brightness of the staining. It has become an integral part of the staining procedure for many laboratories when doing a Masson's trichrome, and is often used for other similar techniques as well. The active component in this form of secondary fixation is the picric acid, and sometimes a simple aqueous picric acid solution is used rather than the mixture. Mostly this is due to the formalin content which gives off excess fumes when heated and which many of us would rather avoid.

Lendrum and his co-workers have recommended a procedure based on this for their trichrome methods for fibrin, but used picro-mercuric-ethanol, that is, ethanol saturated with both picric acid and mercuric chloride.

Just to emphasise, the treatment of sections with picric acid or Bouin's fluid, or Lendrum's picro-mercuric-ethanol, is a form of secondary fixation. It is not mordanting.

Tissue accessibility

The component of tissue with which we are concerned is primarily the proteins. These are built up of a series of amino acids, so called because they have both an amino group and a carboxyl group in a single molecule. Note that these two groups are involved in the peptide linkage by which amino acids join to form polypeptides, and they are therefore not available for attaching to dyes. They do not participate in staining.

Amino acids form chains through peptide linkages. As they do so the chain tends to make a spiral due to the placement of the amino and carboxyl groups on each amino acid. That spiral then tends to bend as different groups attached to the chain come close to each other and are attracted to, or repulsed from, each other in a variety of ways, whether that be ionic or by non-polar forces. The subsequent bending causes other groups to come in contact, and so it continues until we get a very convoluted molecule with attractions and repulsions between various groups inside the areas that are folded and which are thus hidden from reactions. Some groups are on the outside of the folded structure, however, or very close to the surface. It is these groups that are responsible for staining rather than those deep within the folds.

Before the dyes can attach ionically to the tissue amino groups, they must first get close enough for the reaction to take place. The folding of protein molecules inhibits many of the amino groups from participating in the reactions as they are inside the folds. Think of it as a large loosely packed skein of wool. The material on the outside is contacted immediately, but the material beneath the surface may require some time to become involved, and the deepest layers at the center of the skein may never be contacted. Time is required.

However, before we get to stain these proteins we apply chemicals like formaldehyde, mercury salts, picric acid, acetic acid and so on, in fact a variety of chemicals used during the fixation, dehydration, and clearing processes. Then we add insult to injury by heating them in a bath of molten paraffin wax and denature them with heat as well as with chemicals. The end result is that it is very difficult, if not impossible, to determine the exact structure at a molecular level of the proteins we are staining. We can only deal in mass action explanations because we have no real idea what the actual molecular structure is.

In addition, we must also be aware that there is an organised aspect to the structure. We are not just dealing with a mass of folded proteins, we are also dealing with lipoproteins, proteoglycans, fibres, membranes, exudates, coatings and so on. These just complicate the subject.

There may also be other factors which inhibit access to the tissue. For instance, erythrocytes have a

lipoprotein membrane surrounding them, usually called the red cell envelope, inside of which there is an amount of loosely organised hemoglobin. The hemoglobin itself may not be a problem to stain, but getting the dye through the lipoprotein envelope may be. The function of the red cell envelope, after all, is to restrict the contents from leaking out. It is structured in such a way as to be an impenetrable barrier, except to the gases being exchanged. It should come as no surprise that it would inhibit access by the dye, even though the contents on the other side of the membrane are easily stained. Some means of overcoming the fence-like nature of the envelope would be very useful.

It has also been suggested that many fibres have some lipid associated with them. These lipids tend to be hydrophobic and may resist the action of aqueous solvents. When a dye is applied in water, it must first overcome the hydrophobic nature of the protein. Staining is not stopped, but it may be less intense than it would otherwise be. Less dye would attach to the protein as a consequence or, if there is a lot of hydrophobic lipid, staining is inhibited so much that the target material is not demonstrated. This often involves fibrin, which may trap all sorts of materials within itself when it forms, including lipid material circulating in the plasma, from which fibrin is derived. Since fixatives are often applied initially in aqueous solution, it is possible that the fixation process may also have been inhibited by this lipid, and the proteins may not have been adequately fixed.

This issue was addressed by Lendrum and his coworkers with respect to their trichrome methods for fibrin. They strongly recommended initial fixation in mercuric chloride, preferably formal sublimate, for an extended period of 7-10 days depending on the tissue. Following processing and sectioning the sections were to be dewaxed then soaked in trichlorethylene at 56°C for 48 hours to remove all residual lipid, then the section was refixed with picro-mercuric-ethanol for 24 hours. The mercury pigment was removed and the trichrome stain done. This procedure works very well and produces very beautiful stained sections, but it is hardly suitable for a routine diagnostic laboratory. Many of us used to just soak dewaxed sections of formalin fixed, paraffin embedded material in Bouin's fluid or saturated aqueous picric acid for an hour or two before staining, mostly with quite adequate results.

Tissue texture

Sometimes the tissue just has so much material packed into such a small volume it is difficult for the dye to get to it. Tendon, for instance, sometimes just doesn't stain. That is an extreme, but it illustrates the point that some tissues may be impenetrable by the dye just because the fibres or other material from which it is made are so physically closely packed together. This tissue may, or may not, have sufficient amino groups ionised for adequate staining, but because the dye can't get to them they do not stain or, if they stain the reagents we need to use afterwards can't modify their attachment as we would usually expect.

This can be overcome to some degree with alcoholic solvents, and tendon will sometimes be coloured yellow in fibrin staining methods as a consequence. It is also possible to heat the solution to increase staining, but this has no application that I am familiar with.

There is another side to this coin. This other side of the coin in this context is that we sometimes talk about tissue components being "loosely textured", that is they are easily penetrated by dye solutions. Collagen can be one such loosely textured material, particularly areolar connective tissue, and it is consequently easily stained, taking the last dye applied. Of course, there must be a whole range of textures between these two extremes.

Tissue reactivity

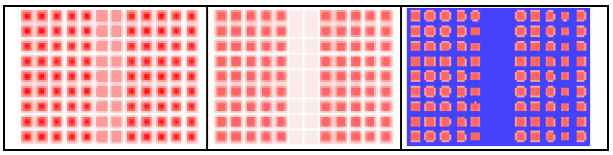
Lets assume for a moment that the tissue amino groups are completely accessible to dyes. We apply the dye in acidified aqueous solution. There is no inhibition, and every amino group takes up a dye molecule. We have maximum staining. Still we see that some parts of the tissue are more darkly stained than others. Why is this?

Obviously, the differences must be due to more dye attaching to some parts of the tissue than to others. The inference is that some tissues must contain more amino groups per unit of area than others do. In other words they are more reactive tissues. This is sometimes referred to as tissue density, i.e. the density of reactive amino groups. I am not sure that expression is the most appropriate because it can be confused with tissue accessibility due to the tissue texture and close physical packing of structures. The term "reactive density" is perhaps more appropriate.

What is the effect of tissue reactivity? Consider a tissue in which one part has 10 amino groups per area and another which has five amino groups per area. We then apply some means of removing the dye evenly from across the whole tissue at five amino groups from each area. The result is that one area becomes unstained, the other still has half the dye

attached. The dye applied following that will then colour the area that is now unstained, resulting in a distinct colour contrast.

In real life staining it is usually not as cut and dried as that, but it serves to illustrate the point. In the diagram below the grid on the left has darkly and weakly red stained cells. The middle grid shows the effect of removing an equal amount of dye from each cell in the grid. The grid on the right illustrates the results of then applying a contrasting colour to stain all uncoloured material.



The Dyes

Acid dyes are quite a diverse group, and there are lots of them, although we tend to use a limited number over and over in different ways. Since they all depend on ionic attachment to amino groups on the tissue via their hydroxyl and carboxyl groups predominantly, they are all very similar in their action. It is easy to demonstrate that an acid dye will stain nearly all tissue components simply by dipping a section into an aqueous solution for a few minutes. With few exceptions everything stains. So how then can we colour different tissue components in contrasting colours when all the dyes will attach to the same tissue groups? The answer is that we must manipulate the dyes by means of their differences from each other and the physcial differences between various tissue types.

Dyes do vary. An example is their formula weight and presumably differences in formula weight also means differences in physical molecule size and shape. They differ in the number of ionising groups and in their in solubility in water and other solvents as well. Unfortunately, many of these characteristics of dyes are poorly documented. Many of the explanations are from the dim and distant past of the 1950s, when explanations were given to explain what was going on without any scientific proof. Subsequent successes in the manipulation of the staining process have tended to validate those hypotheses, but there is still little scientifically validated proof. Very few peer reviewed papers have been published in this area even though the explanations are generally accepted and methods based upon them successful.

Dye solubility

Dyes vary in how much will dissolve in a solvent. Usually more will dissolve in water than ethanol, but this is not always the case. As a general rule, with stronger solutions for any given length of time, more dye will attach to the tissue. The amount tends to be limited as the usual reaction is that dye attaches to a tissue amino group ionically. When all of the amino groups are used up, no more dye can attach. Whether we do this with a 5% solution for 5 minutes or a 1% solution for 30 minutes, the end result is likely to be the same. For that reason we usually do not use solutions which are saturated with a dye, with a few exceptions targeting erythrocytes usually.

We should also keep in mind that, although ionic bonding is the norm, there are exceptions to this as well and both van der Waal's forces and hydrogen bonding have been implicated in some types of staining Techniques that depend on this type of staining usually involve alcoholic solution and alkaline pH, but not always.

Dye aggregates

Dyes are relatively large molecular structures with formula weights ranging from about 300 to over 1,000. One characteristic of these structures is that they tend to aggregate in solution due to van der Waals forces. These, as you will be aware, are the transient attractions between molecules that arises due to where the electrons tend to be situated in the molecules at any given time as they orbit the atoms. It is probable that larger molecules tend to form aggregates with more molecules in the aggregate than do smaller molecules, i.e. the number of molecules in an aggregate is non-linear and the larger the size of the molecule, the more of them will be attracted together.. Unfortunately we have no means of measuring the size of these aggregates, so it has become the practice to refer to the dye's molecular weight as a substitute. Keep in mind, though, that the molecular weight does not correlate directly with the dye aggregate size. Nevertheless, choosing dyes based on their molecular weight does seem to work quite well.

It is also known that van der Waals forces vary depending on the solvent used. Water may be considered the standard as a strongly polar solvent. It owes its unique characteristics, including its solvent abilities, to the hydrogen bonding between its molecules, another force deriving from the

positioning of electrons on each molecule, but this time somewhat more permanent. In water, the aggregates tend to be larger than in other solvents due to its polarity. In less polar solvents such as ethanol or 2-ethoxy-ethanol (cellosolve) to name the two commonest, dye aggregation is weaker and the aggregates physically smaller.

This characterisitic can be exploited by applying a dye in one solvent, then changing the solvent to wash out the excess. It is usually used with small molecular weight yellow and orange dyes such as orange G and martius yellow to stain erythrocytes in contrast to fibrin. Methods such as the MSB and the picro-Mallory use this approach. The erythrocytes are stained by applying a strong solution of a small molecular weight yellow dye in alcoholic solution. Such a solution will have a small dye aggregate size, and the smaller aggregates will be able to pass through the limited size spaces in the red cell envelope. The alcoholic solution is then washed off with water for long enough that the water can penetrate the red cell envelope as well and replace the ethanol. As it does so, the dye forms larger aggregates because the solvent is now more polar. These larger dye aggregates are too large physically to pass through the spaces in the red cell envelope and are trapped within the red cell.

That does not mean the yellow dye cannot be washed out eventually, even with water. We must remember that van der Waal's forces are transient. That means the aggregates form, break up and reform continually. At some times the dye aggregates are small enough to pass, so over a period of time all the dye can be washed out. However, it takes much longer than it would if the washing were done with ethanol and the aggregate size were always smaller. This approach should be considered a method useful to delay extraction of dye from erythrocytes thus giving an opportunity to stain other material in contrasting colours.

Accentuation and Solvent pH

Accentuators are reagents used to increase the intensity of staining reactions, although they are occasionally used to inhibit staining of one tissue component in order to emphasise another, in amyloid staining with direct cotton dyes from an alkaline medium, for instance. Reagents applied to inhibit staining are sometimes referred to as a "***resist***" rather than an accentuator, since their function is to cause the tissue to resist being stained.

The commonest means of accentuation is to adjust the pH to give maximal attachment of the dye to tissue. Generally, an acid dye from an acid solvent will intensify staining intensity. Similarly, applying acid dyes from an alkaline solvent will usually inhibit staining.

To a limited degree, the level of increase or decrease in intensity depends on how great the pH change is. Eosin, for instance, is often used in "mildly" alkaline solution with a pH of around 7.5 or so. Anything much more than that inhibits staining so much that it does not give enough colour to the tissue. Also, as already mentioned, adding acetic acid to an eosin solution results in distinctly brighter but more homogenous staining.

Often the intent is simply to maximise the depth of colour with an acid dye, to cause the maximum dye to attach. This is much simpler, and just requires that an acid is added in sufficient quantity to bring the pH to about 3-4. Most commonly this is obtained with a 1% or 2% solution of acetic acid. That particular pH is important as tissue amino groups are most reactive at that pH. It is the tissue amino groups that bind acid dyes, remember, so the more of those that are able to react, the better. Of course, sufficient time and dye concentration have to be allowed for the attachment to take place fully.

In van Gieson's method, and similar solutions, the necessary pH is supplied by the picric acid. In Masson type procedures, and in one-step methods, it is usual to add acetic acid for the required pH. Yellowsolve techniques usually do not include an acid with the differentiating yellow dye, although it may be included with the red plasma stain.

It is predominantly the tissue that is affected by the pH. The affect on the dye is less obvious, but it is also affected. In acid dyes the ionising groups are usually hydroxyl and carboxyl, and the ionisation of both is depressed in acid pH. We could argue that using acid dyes in a low pH solution should inhibit staining, but it clearly does not. That is likely due simply to the number of molecules involved. A solution of an acid dye will always have some negatively charged groups present and the sheer number is sufficient to ensure maximum attachment to the maximally ionised amino groups. In other words, the pH of the medium seems to affect the tissue components more than it does the dye.

Dye vigour

Dyes vary in the speed with which they stain tissue. A few seconds in some is enough for distinct colouration, minutes in others and the staining is minimal. This is dye vigour, or dye reactivity. There is almost no data about it, although there is an ocassional mention in passing. It is sometimes noted that smaller molecular weight dyes are more vigorous than larger molecular weight dyes, although I do not believe that is an absolute rule. I would presume that the number of ionising groups a dye has may have an effect on how rapidly it stains but, again, there is no substantive data. Most of us, though, will be aware that some dyes stain more efficiently than others.

Displacement

When I was at school, about a million years ago, getting ready for my "O" level chemistry, we studied a phenonemon called "double decomposition". The classic example given was the addition of a solution of silver nitrate to a solution of sodium chloride. The anions and cations reacted with each other, changed places and silver chloride precipitated out as a white cloud.

The principle is that elements can substitute for and replace each other, silver for sodium in the instance given. So can dyes. If we apply an acid dye to a tissue for a few minutes it will attach to all the amino groups available. If we then rinse it off and apply a different acid dye, the second dye will begin to replace the first dye and itself attach to the amino groups, until it eventually completely replaces the first dye. This is called "***displacement***", as the second dye displaces the first dye.

Like most of these processes, though, this does not happen instantaneously. It takes some time for one dye to completely replace another at every amino group. It is quite feasible to remove the second dye part way through the displacement process so that both dyes are colouring the tissue. Usually the results are not that clearly defined, and it not used as a staining technique much with aqueous solvents. Note, however, that it is the basis for the yellowsolve techniques, including the HPS, Lendrum's phloxine-tartrazine, the fuchsin-miller and the methods that derived from it.

It is sometimes assumed that the larger molecular weight dyes will replace smaller molecular weight dyes but not the other way around, so we must use a molecular weight sequence of small, intermediate and large. This is not so. In his yellow collagen variant, Masson used acid fuchsin for muscle and metanil yellow for collagen. Metanil yellow has a lower molecular weight than acid fuchsin, indicating that acid dyes can displace each other whatever their relative molecular weights. The relevance of molecular weight is with regards to aggregate size, nevertheless, it is true that the common sequence is indeed small, intermediate and large molecular weight dyes applied in that order.

Polyacids

Many trichrome methods use polyacids to increase contrast, although they are often targeted towards the contrast between collagen and muscle, usually smooth muscle. The two commonest polyacids are phosphomolybdic acid (molybdophosphoric acid) and phosphotungstic acid (tungstophosphoric acid). There is at least one method, a variant of the picro-Mallory that uses trichloroacetic acid, and I do recall coming across a method that used tungstosilicic acid on one occasion.

It is sometimes stated that only phosphomolybdic acid and phosphotungstic acid are suitable in this role, but the recommendation that trichloroacetic acid can be used in a picro-Mallory variant makes this clearly untrue. There is no doubt however, that they are the most common. It is also sometimes stated that phosphotungstic acid gives sharper results that phosphomolybdic acid, but I have never been able to detect any difference and I quite happily use either one. I also substitute one for the other if I do not have the specified one available, and I have never noticed a problem.

I draw attention to the trichloroacetic acid because of a theory in the United States about how the phosphomolybdic and phosphotungstic acids work. It holds that the metal component (molybdenum or tungsten) acts "like a mordant". That is, the polyacid deviates into the collagen or other structures and then the subsequently applied dye chemically links to it in some fashion through the molybdenum or tungsten. The presence of residual amounts of molybdenum or tungsten following staining is pointed to as proof that this takes place. I do not subscribe to this theory. I would expect residual phosphomolybdic acid and phosphotungstic acid to be present since the processes involved are not allowed to go to conclusion. We interrupt them when the effect we want has been obtained, remove the reagents but leave some of each reagent still in the tissue. There is also no explanation of the chemistry of this mordant-like effect which leaves an acid dye acting as a large anion after being mordanted, when other mordant dyes behave as large cations after mordanting, and attach to negatively charged

structures rather than the positively charged amino groups that are stained in this case.

My point is that trichloroacetic acid can fulfill the same function as phosphomolybdic acid and phosphotungstic acid but does not contain any metal. It cannot therefore form a link with an acid dye through a metal component, and cannot behave as a mordant. If trichloroacetic acid can successfully carry out the function yet clearly not mordant the dye, then it would seem to me that the explanation is very unlikely to be so for the other two polyacids.

So how do they work? Probably the best way of viewing polyacids is to think of them as very large molecular weight dyes, but colourless. They have very large molecular weights, but have overall negative charges. Phosphomolybdic acid has a molecular weight of 1825 and phosphotungstic acid has a molecular weight of 2880. Trichloroacetic acid has a molecular weight of 163. The last is interesting, in that a molecular weight of 163 is not particularly large in comparison to the other reagents and less than most dyes. It calls into question whether the molecular weight has that much to do with it, in other words, whether a high molecular weight reagent is necessary. I refer you back to my comments about the sequence of small, intermediate and large molecular weight dyes. Perhaps in multi-step techniques the ***sequential application*** of the dyes to displace each other is more important than the relative sizes of the molecules.

Staining order

After all that detail, what does it lead to? In most cases it is generally accepted that tissues are most resistant to displacement in the following order:

- Erythrocytes
- Eosinophil granules
- Keratin
- Fibrin
- Muscle
- Cytoplasm
- Bone and tendon
- Collagen
- Areolar connective tissue

Nuclear staining

Early methods using acid dyes did not prestain the nuclei, so the chromatin was usually stained by the first acid dye applied, acid fuchsin in a Mallory, or azocarmine G in Heidenhain's Azan method. Subsequent methods incorporated some means of prestaining nuclei with a dye that could resist extraction by subsequent acid treatment. This was usually an iron hematoxylin, weigert's being the most common, although there are several others, some of which are stable for several weeks. Iron hematoxylins are reported to stain nuclei black in trichrome methods. I have rarely seen that. I interpret the colour to be a dark grey-brown and not particularly appealing.

Some people prefer to use a sequence stain of iron alum-celestine blue followed by a progressive hemalum. This effectively duplicates an iron hematoxylin, as the hematein replaces the celestime blue attached to the nuclei in the first stain. In fact some people merely use an acidified iron alum without celestine blue initially followed by progressive hemalum, with identical results.

Multi-step trichrome

Multi-step trichromes are those in which we sequentially apply dyes to the tissue. The initial methods of this type was the Mallory type. A simple solution of acid fuchsin was applied, then briefly washed. A mixture of aniline blue (methyl blue) in mixture with orange G was then applied and stained the collagen blue and erythroyctes orange. The disadvantage of this technique was that the acid fuchsin was applied in simple aqueous solution with no added acetic acid to accentuate it, and the dye did not stain very intensely. The brief wash afterwards was often enough to remove most of the dye from the muscle, so that when the blue dye was applied it stained both collagen and muscle. Although useful, this difficulty detracted from its popularity.

When Masson's technique was introduced the situation improved tremendously. Masson's method includes acetic acid in the dye solutions, so attachment is optimal. The nuclei are also prestained so they contrast clearly with the red of the muscle. Masson actually recommended four variants in his method. One used acid fuchsin alone, two used mixtures of acid fuchsin and xylidine ponceau, and one used xylidine ponceau alone. All used a saturated solution of aniline blue in dilute acetic acid. Aniline blue was a mixture of water blue and methyl blue, and it can now be either one of those or a mixture. Masson also published two other variants. They both used acid fuchsin, but one stained collagen with a 0.5% solution of aniline blue with phosphomolybdic acid, the other used metanil yellow instead of aniline blue.

The Masson type of stain now follows the following pattern.

1. Bring sections to water with xylene and ethanol.
2. Refix with Bouin's fluid or saturated aqueous picric acid at 56ºC for 1 hour.
3. Wash with tap water to remove the yellow colour.
4. Stain nuclei with an acid resistant method:
5. Apply the plasma stain for five minutes or so, until everything is dark red.
6. Rinse with distilled water or dilute acetic acid.
7. Apply the polyacid for five minutes or so until collagen is pale but muscle is still red.
8. Rinse with distilled water or dilute acetic acid.
9. Stain with the fibre stain for five minutes or so until collagen is stained darkly.
10. Rinse with distilled water or dilute acetic acid.
11. Dehydrate with ethanol, clear with xylene and mount with a resinous medium

So what is going on? The plasma stain, a combination of red acid dyes, stains all the tissue deeply. The polyacid is then applied and displaces the plasma stain from amino groups of the most accessible tissue. This is the collagen which gives up the red dye soonest. When enough has been displaced the polyacid is removed and displacement of the plasma stain stops. The fibre stain is then applied, blue or green, and displaces the polyacid from the collagen, staining it in the process. The role of the polyacid is that of both a differentiator and a dye resist, since the fibre stain must first displace the polyacid before it can further displace the plasma stain. When collagen is stained darkly enough, but muscle is still red, the fibre stain is washed off. The results show collagen as blue or green in contrast to red muscle and epithelial cells, with distinct erythrocytes, depending on the particular combination of dyes used.

Masson's original variants (Gray 1954)

	Ingredient	Var 1	Var 2	Var 3	Var 4	Var 5	Var 6
Plasma Stain	Acid fuchsin	0.5 g	0.35 g		1.0 g	0.1 g	1.0 g
	Xylidine ponceau		0.65 g	1.0 g	1.0 g		
	Acetic acid	0.5 mL	1.0 mL	1.0 mL	1.0 mL		1.0 mL
	Distilled Water	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL
Polyacid	Phosphomolybdic acid	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g
	Distilled Water	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL
Fibre Stain	Aniline blue	Sat.	Sat.	Sat.	Sat.	0.5g	
	Metanil yellow						Sat.
	Acetic acid	2.5 mL	2.5 mL	2.5 mL	2.5 mL		
	Distilled Water	97.5 mL	97.5 mL	97.5 mL	97.5 mL	100 mL	100 mL
	Phosphomolybdic acid					1.0 g	

Rinse variations 1-4 & 6 with 1% acetic acid. Stain variation 5 at 50°C

There are other trichrome methods of this type. Lendrum[(*Lendrum, et al 1962*)] and his coworkers published several different methods targeting fibrin, a feature of which is the contrasting colouration between yellow stained erythrocytes and red fibrin. The picro-Mallory is the gold standard for these techniques, but the MSB – martius, scarlet, blue – is extremely popular as a fairly easy and reliable technique, whereas the picro-Mallory tends to be quite dependent on the stainer's skill.

Both of these methods use small molecular weight yellow, acid dyes saturated in ethanol to stain erythrocytes yellow. Both then wash with water to change the solvent and to remove the yellow dye from the other tissues. The staining from that point on follows the standard Masson pattern, but usually uses phosphotungstic acid. The purpose in going to so much trouble to differentiate between erythrocytes and fibrin is for renal biopsies. In some conditions small fibrin deposits in glomeruli can resemble

erythrocytes and a distinct colour difference can help with evaluation.

Lendrum also published some more obscure methods for fibrin. He believed that once it had been deposited, fibrin matured slowly until it could not be distinguished from collagen. His methods attempt to stain fibrin in distinction to collagen and erythrocytes at different stages in this conversion process. The picro-Mallory demonstrated young to middle aged fibrin red, with really fresh fibrin often stained orange. The MSB stained middle aged fibrin red, the Masson 44/41 demonstrates older fibrin, and the OBDR4 or Obadiah, stained the oldest fibrin blue in contrast to red collagen. These latter two methods were often less than conclusive, but the picro-Mallory and the MSB both were standard techniques before immunological methods came along and replaced them.

Lendrum's group(*Lendrum, Slidders & Fraser 1972*) also introduced an approach to trichrome staining in which small molecular weight acid dyes, yellow, orange or red, were applied in ethanolic solution, washed with water, then displaced with larger molecular weight, yellow, red or blue dyes. Of course, the particular dyes to be used were chosen to give contrast – two yellow dyes would appear to be pointless, except that is what Lendrum recommended following a PAS. The technique was standardised as a few minutes in the ethanolic solution, rinse well with distilled water, then displace with the aqueous solution for half to two hours. The results, of course, varied depending on the dyes chosen. This approach never caught on, partly because experimentation was needed before technologists could choose appropriate combinations, but also because the dyes were not always readily available.

An Australian named Shoobridge extended this concept of standardised trichromes considerably. His solutions were standardised in concentration and pH. The technique was recommended for automated systems. However, it is quite a long procedure and never caught on.

In addition to the trichrome methods for differentiating collagen and muscle, or differentiating erythrocytes, fibrin and muscle, some other specialised methods have been published. Slidders' OFG(*Slidders 1961)* (Orange, Fuchsin, Green) is a typical trichrome targeted towards differentiating between acidophils and basiphils in the pituitary, and Dawe's(*Dawes 1972)*,method targets fetal tissue. There are a fairly large number of these methods, differing predominantly in the plasma dye used, and for how long the polyacid is applied, and consequently how complete the displacement of the plasma stain is.

All of these methods are very similar. The differences between them tend to be the molecular weight of the plasma stain, and for how long the polyacid is allowed to act and remove the plasma stain. In fact, many of them can be substituted for one another by altering the staining times at each step to emphasise one particular tissue component. There is nothing magical about these techniques: it is all about operator control.

One-step trichromes

The earliest trichromes, perhaps more accurately called dichromes, used picric acid as a yellow dye in conjunction with another acid dye of contrasting colour. With one exception, these methods are not popular today, which is a pity. The exception is the van Gieson stain. Applied following a simple iron hematoxylin such as Weigert's, it very simply differentiates between cytoplasm and collagen, demonstrating the finest collagen fibres well. I found it very surprising when I first emigrated to Canada that the method is only used there as a counterstain rather than a primary method in its own right, Masson's trichrome being preferred. Masson, being as he was a pathologist in Montreal, one of Canada's major cities, may explain that.

Picric acid used to be used with many contrasting colour dyes. As an assignment in a histology correspondence course I used to give on behalf of the Canadian Professional society, I assigned a project which involved selecting several acid dyes of contrasting colours to picric acid, then to use them in van Gieson type procedures on a variety of tissues in a composite block. That is, add 50 mg of each dye to 5 mL distilled water, then add 45 mL saturated aqueous picric acid. The written report required a detailed description of the results. I still recommend this as a learning tool.

The van Gieson itself uses 0.05% acid fuchsin in saturated aqueous picric acid. There are several modifications of this method, differing in the amount of acid fuchsin used (see Appendix 3),. The picric acid in this method likely functions in a dual role, that of an acid to lower the pH, and as a small molecular weight yellow dye. The solution is applied for a short period, and removed when staining is satisfactory. Leaving the solution to stain for a long time eventually results in everything staining red except erythrocytes. Time is always a factor.

It is possible to get results similar to the van Gieson using a small molecular weight yellow, acid dye in conjunction with acid fuchsin, or something similar. Squires[(Squire 1892)], in 1892, suggested 0.3% acid fuchsin in 2% orange G for a couple of minutes, for instance. These tend not to be worth while, except as replacements for simple eosin counterstains, perhaps. The earliest multi-step trichromes had a component similar to this, but using aniline blue in conjunction with orange G to displace pre-applied acid fuchsin. The orange G was to colour erythrocytes, while the blue was for collagen.

One step trichromes today do not focus on the van Gieson type. They focus on methods which give results resembling the Masson type, often with yellow erythrocyte staining.

One of the most popular is the Gomori one-step technique. This uses a mixture containing chromotrope 2R as the plasma stain, fast green FCF as the collagen stain, and acetic and phosphotungstic acids. Following prestaining of the nuclei with an iron hematoxylin the solution is applied for a few minutes then rinsed off, dehydrated and cleared. The results are similar to a Masson trichrome.

There are several similar methods, some of them also including orange G, such as Mcfarlanes method which has picric acid, acid fuchsin, aniline blue, phosphotungstic and acetic acids. The staining technique is very similar to the Gomori one-step. Results are similar to a full trichrome, with orange erythrocytes, red muscle and blue collagen.

Even Papanicolaou got in on the act and published a one-step technique for sections which had orange G, eosin Y, acid fuchsin and light green, along with both polyacids and acetic acid in an aqueous solvent. Clearly this was not his cytoscreening solution, but we can perhaps see the beginnings of its development here. The cytoscreening Papanicolaou solutions are actually ethanolic two stage one-step trichrome hybrids. Prestaining nuclei with hemalum, followed by orange G for erythrocytes and some cells and a one-step trichrome to finish.

The mechanism behind these techniques is one of "competition" for available amino groups. It depends on the interaction of all those factors I detailed earlier on. In a complex solution, as these are, some reagents will penetrate tissue more quickly than others and bind to any available groups. As time passes other reagents will gain access and displace them. This is an ongoing dynamic process and time is an important factor, since it is a truism that the largest molecular weight dye will eventually stain most of the amino groups. In one-step staining it must be noted that molecular weight is an important factor.

The more vigorous and smaller molecular weight dyes will stain the most accessible tissues immediately and eventually colour much of the rest, including erythrocytes. The intermediate molecular weight dyes will displace them in short order, to be replaced themselves by the largest molecular weight dyes. It is more difficult to explain the role of the polyacids in one-step methods. It is easy to say that the role is the same as in the multi-step methods, but I do not see how it can be. I suspect that polyacids function more as a resist for the dyes than as differentiator, that is, they slow the activity of the large molecular weight dyes and by doing so greater contrast is obtained.

Optimising the staining with a one-step trichrome technique is an important skill so I suggest, as an educational tool, that a complex one-step trichrome solution be made and the staining protocol be optimised in a series of experiments. The first step is to stain several sections for different times. Start at one second, and do doubling of times until overstaining is reached. It requires about 10 or 11 sections to get from 1 second to 16 minutes.

Due to the interaction between the different dyes and the differences that the duration of staining brings about, one-step methods are more highly constrained than multi-step methods. By that I mean that a staining protocol has to be established and followed precisely. Not to do so introduces variability in the end product and the stains become unreliable. When this is coupled with the differences in fixation and pretreatment used in different laboratories, standardising the techniques becomes an absolute must. One-step methods can be very useful, but shine when many similar sections are to be stained.

A learning exercise

It can be an interesting exercise to make up a one-step solution then determine its protocol for use in the routine laboratory staining of kidney biopsies, skin sections or placenta, the particular tissue should be chosen for convenience. Nothing teaches so well as actually doing it.

- Choose a yellow dye from orange G, martius yellow, picric acid or metanil yellow.
- Choose a red dye from acid fuchsin, xylidine ponceau, ponceau 6R or biebrich scarlet.

- Choose a final dye from light green, fast green, methyl blue or water blue.
- Choose a polyacid from tungstophosphoric, molybdophosphoric or trichloroacetic acids.
- Make a 1% aqueous solution of each selected dye.
- Make a 1% aqueous solution of the selected polyacid.
- Combine 1 volume of each solution (12 mL of each will fill a Coplin jar).
- Add sufficient acetic acid for a 1% concentration (0.5 mL if 12 mL volumes were used).

Method

1. Bring sections to water.
2. Treat with picric acid or Bouin's fluid.
3. Prestain sections with iron hematoxylin
4. Stain in the mixture for ??? minutes.
5. Rinse with distilled water.
6. Dehydrate, clear and mount.

The timing and proportions of each dye must now be optimised. The time of application may be altered or the proportion of each component in the solution may be changed. The two are not equivalent.

Changing the time will do two things, the staining is likely to become paler if staining time is reduced or darker if it is increased, and it may alter the colour of each tissue component.

- Increasing staining time will favour the higher molecular weight dyes over the small molecular weight dyes. Erythroctes may be reddened and muscle may be tinged with blue or green.
- Decreasing staining time will favour the small molecular weight dyes over the larger molecular weight dyes. Cytoplasm and muscle may become yellower and the collagen may be redder.

First, an approximate staining time should be set. This can be done by staining several sections for different times. Doubling the staining time from 1 minute to 1 hour would take 7 sections. Staining in 5 minute increments from 5 minutes to 1 hour would take 12 sections. A few more sections could then be stained to get the most appropriate time.

Changing the proportions of each component will affect the relationship between what stains with which dye. It may be done three ways.

- Adding a different amount of the 1% stock solution. This not only changes the amount of the dye being targeted, but also changes the concentration of the other two dyes in the solution due to the change in final volume.
- Changing the concentration of the stock solution and adding the same amount. This permits the relative proportions of the other two dyes to remain unaffected, but is wasteful of dyes and time consuming.
- Starting with strong stock solutions, but standardising the final volume of the mixture by adding water or dilute acetic acid. This enables any of the ingredients to be altered without affecting any of the others providing that the final mixture volume is not changed.

The following effects can be expected to some degree, but it must be understood that the changes may or may not not be distinct. This is not an exact process and depends a great deal on personal evaluation.

- Increasing the yellow dye will cause it to resist the staining by the red dye. Cytoplasm and muscle may become yellower.
- Decreasing the yellow dye will cause the red component to stain more effectively with little change to the blue. Erythrocytes may become redder.
- Increasing the red dye will cause it to replace the yellow dye more and better resist staining by the blue dye. Erythrocytes may be redder and there may be some red retained in the collagen.
- Decreasing the red dye will cause it to replace the yellow dye less and resist the staining by the blue dye less effectively. Erythrocytes will be yellow and there may be some yellow in cytoplasm and muscle. Collagen may be bluer.
- Increasing the blue dye will cause it to replace the red dye more. Cytoplasm and muscle may become blue tinged.
- Decreasing the blue dye will favour the action of the red dye and collagen may retain some red.
- Increasing the polyacid may resist the blue dye more, so collagen may become red or there may be an increase of poor colouration in more inaccessible tissues like bone and tendon.
- Decreasing the polyacid may cause less distinct staining with a degree of colour mixing.

The most effective way is to first set the staining time to bring about the desired depth of colouration, then to adjust the colour balance by altering the proportion of each dye, starting with the yellow, then the red and finally the blue. This procedure sounds far more complicated than it is, and it usually does not take too many changes to set the balance. Once set, the balance and time do not need to be altered until a new

solution is made, and that usually works much the same as the old solution.

One other point must be noted. In the case of the one-step MSB, the combined solution must be left a few days before it will stain correctly. Keep this in mind when making a new combination of dyes. Also, not all combinations are compatible. Dyes are chemicals, after all, and can react with each other. Some combinations may be unstable, or have a short working life.

Yellowsolve methods

The term "yellowsolve" is derived from a contraction of "yellow" and "cellosolve". Cellosolve is the most comment solvent for the yellow dyes used, although the earliest method used ethanol, as in the HPS.

Cellosolve is a solvent which is more correctly known as 2-ethoxy-ethanol or ethylene glycol monoethyl ether. It is inflammable and explosive at temperatures higher than 44°C. It should be used in well ventilated areas and pregnant women should avoid inhaling it. Consult the MSDS.

These methods use an aqueous red dye to overstain the tissue then the section is thoroughly dehydrated and immersed in a yellow dye in a low polarity solvent. The yellow dye displaces the red dye slowly, and the section is removed when the required degree of displacement has occurred. You may recognise here the basic method for the HPS or HES, Lendrum's phloxine tartrazine, the Slidders' fuchsin miller and others. The name appears to come from Lendrum[(2)] who published two methods for fibrin in his paper about kidney fibrin staining and named them Yellowsolve I and Yellowsolve II, although they never became popular. In fact, yellowsolve methods as a group are not all that popular with perhaps the exception of phloxine tartrazine.

The most critical component with these methods is that the low polarity solvent be completely anhydrous. Even a trace of water causes the method to fail, and this cannot be stressed enough. The two solvents usually used are absolute ethanol and 2-ethoxy-ethanol or cellosolve. If you use one of these methods and results are not sharp, check the solvent first and remove any trace of moisture.

In a low polarity solvent dye aggregates will generally be smaller and displacement will generally proceed at a slower pace, sometimes taking hours. As with all acid dye staining methods fixation is critical and mercury is the preferred fixative, but secondary section fixation with picric acid will often do. Do make sure all yellow from the picric acid is removed before staining, though. Some methods, the HPS for instance, are quite satisfactory following formalin fixation.

Displacement follows the usual order, with collagen being displaced first followed by muscle. There is a difference, however, with erythrocyte staining. In most aqueous trichrome methods displacement of erythrocyte staining does not take place due to the large aggregate size. Since yellowsolve methods use low polarity solvents and the dye aggregates are smaller, they can penetrate the red cell envelope easily enough to displace the dye in them at the same time as the collagen is being displaced. For that reason, erythrocytes are often yellow in yellowsolve methods. However, tissues fixed with formalin sometimes do not give up the red from the erythrocytes easily, so prestaining erythrocytes with a yellow dye in ethanol is sometimes used to help the technique along.

These methods are evaluated by inspection and they do not lend themselves well to hard timed protocols. The displacement time varies so much with some of them, quarter of an hour to four hours for the fuchsin miller, that visual inspection is the only reasonable approach. It also explains why they have not become more popular, because they can be quite striking with bright red fibrin strands on a completely yellow background, for instance.

As the displacement is progressive, these techniques can be used for staining fibrin or muscle in contrast to collagen by shortening the displacement period and removing the section from the yellowsolve solution at the appropriate time.

In order to increase contrast, some yellowsolve methods also incorporate a polyacid. It is more common with those variants which target fibrin than those targeting other tissues. The polyacid functions in the same way as it does in a multi-step trichrome, differentiating the collagen and acting as a resist.

Quad and pentachrome methods

Quad and pentachrome stains are not distinct types of acid dye staining methods. They are methods which incorporate staining of other materials into existing methods. In the case of quad stains the parent method is usually a trichrome, and in the case of pentachromes it is usually the HPS.

In quad stains the extra material stained is often elastic, either using orcien or verhoeff's method. Some methods prestain the elastic, some add the orcien to the staining solution. Many variations have been tried with varying success rates. The major problem is that the clarity obtained with a trichrome is often lost due to increased staining of the background. Compare, for instance, a simple van Gieson with a Verhoeff van Gieson.

The most well known pentachrome method is the Movat stain. There are a couple of very good modifications of this. The extra material stained is mucin with alcian blue, followed by an HPS. These methods give very nice appearances, but have to be dealt with individually.

I am mentioning these techniques here because they are often referenced with trichrome methods, but what I have said about trichrome and yellowsolve methods in general applies as much to them, with the added necessity to evaluate the extra stained material.

Conclusion

All acid dye staining depends upon the same thing, the attachment of negatively charged groups on dyes through ionic bonding to positively charged amino groups in the tissue. In order to bring about tinctorial and contrast differences, and to demonstrate different tissue constituents in different colours several strategies are used, the most important of which is the use of displacement, the ability of one dye to replace another dye that has ionically bonded to a tissue constituent.

Differences in dye reactivity, the solvent used and its effect on dye aggregate size, and the length of time the dye is applied all play a part, as do tissue fixation, the accessibility and permeability of particular tissue components, the number of amino groups available in any given structure, and the use of polyacids to differentiate and resist. Manipulating these characteristics of the dyes and the tissues are what makes these methods so useful. I hope I have been able to make the process more comprehensible.

Appendix 1

Staining pretreatment with Bouin's fluid

1. Dewax and hydrate section.
2. Place into Bouin's fluid in a Coplin jar at 56°C for one hour.
3. Wash well with water to remove yellow staining.
4. Continue with the stain.

Saturated aqueous picric acid may be substituted for the Bouin's fluid.

Appendix 2
Trichrome Staining Methods

In the following charts it is to be understood, unless otherwise specified, that paraffin sections are to be brought to water via xylene and ethanols, and an acid resistant nuclear stain applied prior to staining. After staining the sections are to be dehydrated with ethanol, cleared with xylene and mounted with a resinous medium.

It should also be noted that most trichrome methods benefit from fixation with picric acid or mercuric chloride containing fixatives. If simple formalin fixation has been used, then treatment with aqueous picric acid or Bouin's picro-acetic-formalin mixture at 56°C for an hour or so will usually be beneficial

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Terms

Dyes	To conserve space, dyes and chemicals are identified by abbreviations. These are explained on the following page.
Variant	The individual published method. More complete details, including times, are given in individual pages for each method at ***http://stainsfile.info***
RBC	Some trichrome methods incorporate a step for erythrocytes. The commonest colours to use are orange and yellow. Often the dyes are dissolved in ethanol, and may include a polyacid or some other acid.
Plasma	In many trichrome methods the plasma stain is the red component. This step usually demonstrates muscle, cytoplasm and fibrin. If the RBC step is omitted, erythrocytes usually stain with this colouration.
Diff	Many techniques incorporate a differentiation step. Most commonly this uses phosphomolybdic or phosphotungstic acid. The polyacid removes the plasma stain from collagen. This increases the contrast between the plasma and fibre stains.
Fibre	This step incorporates a large molecule dye to stain collagen and bone in a colour that strongly contrasts with the plasma stain. The commonest colours are blue or green. Rarely, yellow is used.
Yellow	Yellowsolve techniques use a large molecular weight yellow dye in an anhydrous solvent. The earliest method used absolute ethanol, but the more complex methods use 2-ethoxy-ethanol (cellosolve). These techniques may, or may not, incorporate a polyacid differentiation step.
Chemical	Reagents other than dyes and solvents.
Wash	A very few methods recommend a final wash in dye solutions. These usually are designed to refresh erythrocyte staining as this may have been degraded by the end of the method, and to remove excess fibre stain.

Abbreviations

Abbr	*Meaning*		*Abbr*	*Meaning*		*Abbr*	*Meaning*
AC	Acetic acid		**EY**	Eosin Y ws		**OG**	Orange G
AB	aniline blue		**FG**	Fast green FCF		**OR**	Orcein
AE	Azo eosin		**FY**	Fast yellow		**OX**	Oxalic acid
AF	Acid fuchsin		**Gly**	Glycerol		**PA**	Picric acid
AZ	Azocarmine		**HCl**	Hydrochloric acid		**PB**	Polar brilliant red BN
BB	Bismarck brown		**HX**	Hematoxylin		**PD**	Potassium dichromate
BF	Basic fuchsin		**IA**	Iron alum		**PH**	Phloxine B
BN	Benzo new blue GS		**LC**	Lithium carbonate		**PL**	Propalan red 3GX
BS	Biebrich scarlet		**LG**	Light green SF yellowish		**PM**	Phosphomolybdic acid
CC	Calcium chloride		**LY**	Lissamine yellow		**PR**	Ponceau 2R
CH	Chromotrope 2R		**LR**	Lissamine fast red		**PS**	Ponceau 6R
CR	Chicago red		**MB**	Methyl blue		**PT**	Phosphotungstic acid
DB	Durazol brilliant blue B		**MG**	Methyl green		**SR**	Sirius red F3B
DW	Distilled water		**MR**	Martius yellow		**SS**	Sodium sulphate
EB	Eosin B		**MY**	Metanil yellow		**SY**	Sun yellow
EE	2-Ethoxy-ethanol		**ML**	Milling yellow 3G		**TA**	Tartrazine
Eth	Ethanol		**NG**	Naphthol green B		**TB**	Toluidine blue
Unna	DW 50, Eth 100% 25, AC 2.5, Gly 10, OR 0.5, AB 0.5						
Taenzer	Eth 50% 100, HCl 1.0, OR 0.8						

Multi step methods

Each column in the chart below represents a separate solution. For each solution, all fluids are to be measured in millilitres and all solids in grams. Unless otherwise specified, the solvent is 100 millilitres distilled water. Where another fluid is included, the volume of water should be adjusted to make 100 millilitres total volume of solvent. Where ethanol is specified, absolute (100%) ethanol should be used. In most cases fluids should be diluted first to make the solvent, then the solids added and dissolved in sequence. All solutions should be filtered before being used.

Variant	RBC	Plasma	Diff	Fibre	Wash	Comments
Brillmeyer		AF 0.2		AB 0.5 OG 0.2 PM 1		
Bensley		AF sat.	PM 1	AB 0.5 OG 2.0		
Crossman		AF 0.3 OG 0.13 AC 1	PM 1	AB 2 AC 2		
Goldner		AF 0.3 PR 0.7 AC 0.2	OG 2 PM 4	LG 0.2 AC 0.2		
Haythorne	OG 0.8 IA 5 HCl 0.06 Eth 4	AF 0.5		AB 2.5 OG 2.5 PM sat.		
Heidenhain's Azan		AZ 2 AC 1	PM 5	OG 2.0 AB 0.5 AC 7.5		
Hollande	OG sat.		PM 1	LG 0.2		Prestain with 1% BF in 70% Eth. The stain is applied in the order of:– diff, RBC, fibre
Koneff	PA 1 OG 0.2 Eth 80	AZ 1 AC 1	PT 5	PT 0.05 OX 2 OG 2		For pituitary cells
Kricheski		AF 0.25		MB 0.3 OG 0.3 PM 0.3		

Laidlaw		AF 1	PM 1	OG 0.25 Eth 70		For acidophil inclusions
Lee-Brown		AF 0.25	PM 1	OG 2.0 MB 0.5 OX 2.0		A simple modification of Mallory
Lendrum & McFarlane	PA 1 OG 0.2 Eth 80	AF 0.5 PR 0.5 SS 0.25 AC 1	PM 1	AB 2 AC 1		
Lendrum Slidders & Fraser	PA 2 Eth 95 Dye 0.5		AC 1 Dye 0.5			RBC-plasma dyes:- AF, AE, MR, OG, PL Fibre dyes:- SY, SR, BN, DB
Lewis & Miller		AF 0.25		MB 0.3 OG 0.3 PM 0.3		For pituitary cells. This is a modified Kricheski's trichrome, but with much longer staining times.
Lillie		BS 1 AC 1	PM 2.5 PT 2.5	FG 2.5 AC 2.5		
McFarlane - A		AF 0.8 PA 0.2 AC 2	PA 1 PT 10 Eth 40	AB 2.5 AC 2.5	PA 0.25 PT 2.5 Eth 10	
McFarlane - B	PA 1.0 OG 0.25 Eth 80	AF 0.25 PR 0.25 AC 1.0	PA 1.0 PT 10 Eth 40	AB 2.5 AC 2.5	PA 0.25 PT 2.5 Eth 20	
Mallory		AF 0.25	PM 1 or PT 1	OG 2.0 MB 0.5 OX 2.0		
Masson type		AF 0.35 PR 0.65 AC 1	PM 1	LG 2 AC 2		A variant often found in reference texts.
Masson - A		AF 0.35 PR 0.65 AC 1	PM 1	AB sat AC 2.5		or PR 1% in AC 1% or AF 0.5% in AC 0.5% or AF 1% & PR 1% in AC 1%
Masson - B		AF 0.1	PM 1	AB 0.5 PM 0.5		
Masson - C		AF 1 AC 1	PM 1	MY sat.		

Masson 44/41		PS 1 AC 1	PT 1	NB 1 AC 1		For old fibrin. Extended mercuric chloride fixation required. Degrease with trichlorethylene. Refix sections with picro-mercuric-ethanol.
Milligan		AF 0.1	PM 1	FG 0.1 AC 0.2		Pretreat, 5 min. with PD 2.25%, HCl 2.5%, Eth 25%. AB can replace FG.
Möllendorf		EY 1 AC 0.3	PM 2	MB 1		
MSB	MY 0.5 PT 2	PS 1 AC 1	PT 1	MB 0.5 AC 1		For fibrin. Extended mercuric chloride fixation preferred. Several other dyes may be substituted. See method
Obadiah	OG 0.5 PT 1	NB 1 AC 1	PT 1	CR 2.5 AC 1 or PB 1 AC 1		For very old fibrin. Extended mercuric chloride fixation required. Degrease with trichlorethylene. Refix sections with picro-mercuric-ethanol.
Patay		PR 1	PM 1	LG 0.5 Eth 90		
Picro-Mallory short	PA sat OG 0.2 Eth 80	AF 1 AC 1	PT 1	MB 2 AC 2		Yellow diff: RBC stain 30, Eth 70 For fibrin. Requires extended mercury fixation for optimal results.
Picro-Mallory long	PA sat LY 0.2 OG 0.2 Eth 80	AC 1 AF 1 or BS 1 or LR 0.2 + AF 0.4	PT 1	MB 1 AC 1		Stock diff: PA 2.5, Eth 100, PT 25 Red diff: stock diff 40, Eth 20, DW 40 Blue diff: stock diff 10, DW 90 For fibrin. Requires extended mercury fixation for optimal results.
Slidders OFG	OG 0.5 PT 0.5 Eth 100	AF 0.5 AC 0.5	PT 1	LG 1.5 AC 1.5		For pituitary cells
Weiss		AF 0.04		AB 0.5 OG 0.2 PM 1		

One step methods

One step trichrome staining methods use a single staining solution, although some may specify pre or post staining treatments. In the chart below all units given for fluids are in millilitres, and all units for solids are in grams. Each reagent should be dissolved into the solvent specified in the second column. Unless otherwise specified, it is suggested that the solvent be made first, then the solids dissolved in it. Filter each solution before use.

Variant	Solvent	Chemical	Dyes	Comments
Cason	DW 100	PT 0.5	OG 1.0 AF 1.5 AB 0.5	
Engel & Cunningham	DW 100 AC 1.0	PT 0.6	CH 0.6 FG 0.3	Gomori's trichrome modified by adjusting pH to 3.4 with 1N NaOH
Gomori	DW 100 AC 1.0	PT 0.6	CH 0.6 FG 0.3	
Kostowiecki	DW 100	PM 1.0	AB 0.06 OG 0.2	Prestain nuclei red.
Ladewig	DW 100	OX 2.0	OG 2.0 AF 1.0 MB 0.5	Pretreat with PT 1% 2 min.
McFarlane	DW 98 AC 2.0	PT 1.0	PA 0.2 AF 1.0 AB 2.0	Post stain wash in PA 0.25% & PT 2.5% in 10% Eth
Papanicolaou	DW 100	PT 0.112 PM 0.225	OG 0.125 EY 0.21 AF 0.1 AB 0.06	This is not the Papanicolaou method used for cervical cancer screening.
Pollak	DW 50 Eth 95% 50 AC 1.0	PT 0.5 PM 0.5	OG 0.25 PR 0.33 AF 0.17 LG 0.15	Add fluids together. Divide into four. Dissolve PM in 1st, PT and OG in 2nd, LG in 3rd, PR and AF in 4th. Add together and filter.
Wallart & Honette	DW 300 AC 2.0	PM 1.0	AF 1.0 FY 1.0	Add AC to 200 DW. Divide in 2. Dissolve AF in one half, FY in the other. Dissolve PM in 100 DW. Combine 30 of each. Filter.

Yellowsolve methods

Yellowsolve methods prestain with an aqueous solution of a red dye, some then differentiate with a polyacid. Then a yellow dye in an anhydrous solvent is applied to progressively replace the red dye in tissues other than the target. In the chart below all units given for fluids are in millilitres, and all units for solids are in grams. Unless otherwise specified, the plasma stain and differentiation solvents are 100 mL of distilled water. The yellow solvent is always an anhydrous fluid.

Variant	Plasma	Diff	Yellow	Comments
Fuchsin-Miller	AF 1.5	PT 1	ML 2.5 EE 100	The target tissue is fibrin. Extended mercuric chloride fixation is required.
Phloxine-tartrazine	PH 0.5 CC 0.5		TA sat. EE 100	The target tissue is acidophil cell inclusions.

Cytoscreening

Papanicolaou	OG 0.5 PT 0.015 Eth 95	LG BB EY PA LC Eth	0.22 0.06 0.22 0.17 1 drop 95	or	0.225 0.05 0.225 0.2 1 drop 95	or	0.045 0.05 0.225 0.2 1 drop 95	or	0.1125 0.05 0.225 0.2 1 drop 95	This is Papanicoloau's cytoscreening method for cervical cancer. The second column is OG6, the other columns are the various EA solutions.

Other methods

In the chart below all units given for fluids are in millilitres, and all units for solids are in grams. It is suggested that the solvent be prepared first, then the solids dissolved in it. Other pre-made solutions which are required may then be added. Pay particular attention to the comments column as other staining steps may be specified.

Variant	Solvent	Chemical	Dyes	Comments
Duprès	DW 200	OX 4.0	TB 0.25 OG 4.0	Pretreat with 1% PM for 10 min TB may be replaced with MG.
Kohashi	Eth 50% 35 Gly 40		EB 0.7 AF (sat aqu) 4 Unna's sol 35	For elastic & collagen pretreat with 0.1% AZ in 1% AC for 15 min. Then in 5% PM for 30-60 min.
Mollier	DW 100		NG 1 AC 1	For elastic & collagen prestain with Taenzer and Weigert's iron hematoxylin, then pretreat with 2% AZ in 1% AC for 15-30 min, followed by 5% PM 2-6 hrs.
Paquin & Goddard	DW 100	PT 0.1	EY 0.07 PH 0.03 OG 0.01	For elastic & collagen prestain with iron HX, and post stain with 0.04% AB in 1% AC
Pasini	Eth 50% 35 Gly 40		EB 0.7 AF (sat aqu) 4.0 Unna's sol 35	For elastic & collagen pretreat with 2% PT for 10 min.
Roque	HCl 0.02M 100		CH 0.5 AB 2.0	For Mallory bodies & collagen prestain with iron HX, then pretreat with 1% PM for 2 min
Walter	Eth 50% 35 Gly 40		EB 0.7 AF (sat aqu) 4.0 Unna's sol 35	For elastic & collagen pretreat with 2.5% IA for 16 hours, then with 2% PT for 10 min.

Appendix 3
Picro-fuchsin variants (Gray 1954)

Variant	Distilled Water	Picric Acid	Acid Fuchsin	Other Comments
Fite	100 mL	0.5 g	0.1 g	Made with: Sat. Picric acid 40 mL, Dist. H_2O 50 mL, 1% Acid fuchsin 10 mL
Hansen	98 mL	Sat.	0.1 g	Add 0.3 mL acetic acid.
Lillie	100 mL	1 g	0.1 g	Add 0.25 mL hydrochloric acid.
Ohlmacher	50 mL	50 mL Sat.	0.5 g	Make by diluting sat. aqueous. picric acid with distilled water
Schaffer	100 mL	Sat.	0.15 g	Add 0.05 mL (5 drops) acetic acid.
Thompson	100 mL	Sat.	0.13 g	
Unna	90 mL	Sat.	0.25 g	Add 0.5 mL nitric acid and 10 mL Glycerol.
Van Gieson	100 mL	Sat.	0.05 g	
Weigert	100 mL	Sat.	0.1 g	
Wilhelmini	90 mL	0.8 g Amm picrate	0.2 g	Add 10 mL 95% ethanol. ***Note:*** ammonium picrate explodes more easily than picric acid.

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