Some routine stains for fresh or embedded plant tissues

Before staining fresh tissue or freshly-made hand sections, look at the tissue without any stain. This will show the overall structure and for many studies gives all the information required. It also allows the native colours of chloroplasts, crystals and specialised vacuoles to be seen. Note that for most studies that only need information about cell and tissue location and orientation, fresh sections are always preferable to killed and embedded tissues because these procedures always distort plant tissues and remove water and solvent-soluble materials.

There is a huge range of dyes and stains that have been used to selectively highlight various cell components. However, there are surprisingly few good comprehensive and up to date reference books outlining types of dyes and stains to use for particular purposes.

Stains for brightfield microscopy

Toluidine blue O

0.05 – 0.1% toluidine blue in ~0.2% sodium benzoate buffer, pH 4.4

Add some stain to a watch glass, then add thin fresh sections for 30 sec to a few minutes, as required. Rinse then mount in water. Toluidine blue O is a polychromatic dye, i.e. it absorbs different colours depending on the nature of its chemical binding with the components of the tissue. It is positively charged at a pH of 4.4. This stains acidic (carboxylated) polysaccharides, like pectin, pink, and compounds containing aromatic benzene rings, like lignin and related compounds, will stain green to blue, depending on their particular chemical structure. In general, unlignified cell walls e.g. of parenchyma and phloem are pink to pinkish-purple and xylem walls are green to greenish blue. However, the thickened walls of lignified fibres are bright sky blue.

Below pH 4, tissues stain only blue or green, since the carboxyl groups on the pectins are uncharged and cannot bind the dye. Only the lignin with its benzene rings will do so, hence the green colour. At a higher pH (11.1), tissues stain mostly dark pink since most (if not all) pectins will be charged. High pH will also hinder the green colour of the lignin.

For resin sections on glass slides, add a drop of stain to cover the sections well, time as required, then rinse the slide in water and cover with a coverslip.

Other options:

0.1% in water
0.1% or less in acidic solution – 50 mM citrate buffer, pH 4.4
50 mM acetate buffer, pH 4.4
0.05% in neutral solution – 100 mM phosphate buffer, pH 6.8
0.1% in basic solution – 1% borax pH 9

PAS reaction/Schiff's reagent

Treat sections in 1% periodic acid for 10 min.
Wash thoroughly in water.
Stain in commercially available Schiff's reagent for 30 sec or longer if required.
Rinse then mount in water.

Schiff's reagent is a solution that will combine chemically with aldehydes to form a bright red product, and is made from pararosanilin treated with sulphurous acid. The periodic acid

treatment oxidises tissue components to produce the aldehydes that the red sulfonated pararosanalin will bind to. Pectins, hemicelluloses and some lignins stain bright red, while cellulose stains faintly. Starch is also stained strongly.

Safranin

Incubate in 1% safranin in water for 1-10 min, rinse, mount in water.

Safranin stains lignified walls, tannins and nuclei red, and will generally stain all tissues if not counterstained with another stain such as fast green – 0.5% in 70-95% ethanol, rinse, mount in water. Fast green stains cellulosic walls and all other cell structures green.

Sudan black B

0.07% Sudan Black B in 70% ethanol

Incubate fresh sections for 15-60 min in stain.
Rinse in 50% ethanol, mount in same

Lipidic components and some hydrophobic polymers stain deep blue-black, remaining walls and cell components stain faint grey.

Neutral red

Stain with 0.01-0.1% neutral red in water for 10-30 min.
Rinse and observe in water

Neutral red stains the cytoplasm and nuclei red in living cells, and at a higher concentration (1%) stains cell walls red in resin-embedded tissues.

I_2/KI for starch

1% iodine crystals (I_2) dissolved in 1% KI (aqueous).

Stain until starch grains turn blue-black.
Rinse and observe in water or observe while in stain.

Iodine stains starch blue-black. If tissue is left too long in this stain, it will turn brown, which reduces the contrast between black starch grains and paler surrounding tissues.

Mäule Reaction: Phenolic/Hydroxycinnamic Acid-Derived Polymer Detection

The Mäule procedure has traditionally been used for determination of syringyl-lignins. More recently, it can be inferred that the Mäule test is also a positive indicator for hydroxycinnamic acid-derived polymers associated with wound healing and suberin deposition.

Incubate fresh tissue sections in 0.5% aqueous $KMnO_4$ solution for 20 min.
Rinse with dH_2O.
Place in 2% HCl for 10 min.
Rinse in dH_2O.
Place in 0.5% ammonia for 5 min.
Rinse in dH_2O and 70% ethanol.
Mount sections in glycerol.

With brightfield microscopy, an orange/reddish-brown to violet colour reaction is considered Mäule positive, whereas no colour reaction is negative.

Cross/Bevan Reaction: Guaiacyl lignins

In the Cross/Bevan reaction, the chlorination of the syringyl nucleus leads to a pink (lignifying cells) or red (lignified cells) colour, whereas the guaiacyl nucleus produces a light (lignifying cells) to dark (lignified cells) brown colour. The mild reaction conditions circumvent the problem of thin tissue section destruction that often occurs during Mäule colour reactions.

Incubate fresh tissue sections in fresh, saturated chlorinated H_2O at 4°C for 10 min.
Rinse 3 times with dH_2O.
Place in 4% sodium sulfite at room temp. for 5 min.
Mount sections in glycerol.

Stains for fluorescence microscopy

Before commencing any work using fluorescent stains, first look at the autofluorescence in unstained tissue. This may give you all the information you need about distribution and abundance of fluorescent cell components and general structure of the cell or tissue type you are interested in. It is also essential to know what the background autofluorescence is before adding a fluorescent stain.

Toluidine blue stain can be used to block autofluorescence. This is also the function of many fluorescence counterstains, such as crystal violet and aniline blue, below.

Rhodamine B EX 556/EM 578

Stain in 0.02% rhodamine B in water for 30 seconds to a few minutes.
Rinse briefly in water, mount in water and observe under UV or blue light.

Hydrophobic walls fluoresce yellowish, other walls fluoresce blueish. In vascular tissues, blue autofluorescence may mask the yellow fluorescence from this stain.

DAPI EX 358/EM 461

Incubate tissue in 0.01% DAPI in water for a few seconds to a few minutes. The fluorescence will increase in intensity the longer the tissue remains in the stain.
Rinse and observe in water under UV light.

DAPI binds into the minor groove of DNA, especially at AT-rich regions. Like PI below, it also binds plant and bacterial cell walls, producing blue to yellowish fluorescence. DAPI is generally membrane permeant, but some cells exclude DAPI and the membranes must be permeabilised before nuclei are stained. Autofluorescence can be blocked by subsequent staining with PAS or toluidine blue. Note that the DNA in mitochondria and chloroplasts is stained with DAPI, as well as nuclear DNA.

Propidium iodide EX 535/EM 617

Tissue is placed in 0.001% PI in water and observed immediately. It will take 5-10 min for PI to penetrate denser tissues.
Observe under UV to green light, fluorescence emitted from orange through to infrared.

Propidium iodide is usually used as a stain for DNA, and it also stains RNA. It works by intercalating into the DNA strand, at a ratio of about 1 molecule per 4-5 base-pairs. However, like DAPI, PI also stains cellulose and lignins very well, presumably intercalating into the cellulose microfibrils and lignin subunits.

Acridine orange EX 460/EM 650 (RNA) EX 500/EM 526 (DNA)

Incubate tissue in 0.01% acridine orange in 5% acetic acid and observe under blue light.

DNA will fluoresce green, and RNA fluoresces red. Acridine orange is usually used as a stain to detect RNA, but interacts with both DNA and RNA by intercalation or electrostatic attractions. Note that condensed chromatin is not efficiently stained by acridine orange.

Calcofluor white EX 350/EM 470

Stock solution 1% calcofluor white in 100 mM Tris buffer, pH 9.0
Working solution 0.01%, dilute 1 in 100 immediately before use.
Stain for 5-10 min, rinse, then observe under UV light.

Calcofluor white intercalates between the fibrils of β-1-4 glucans, such as cellulose, which appear bright white under UV. The stain binds very strongly.

Aniline blue EX 370/EM 509

Stain in 0.05-0.005% (water-soluble) aniline blue in 100 mM phosphate buffer, pH 8.5. High pH is essential. The non-water-soluble dye must be made up in 50% ethanol.
Observe while still in the solution under UV light.

Aniline blue stains β-1-3 glucans such as callose to give a bright yellow-green fluorescence under UV, and at higher concentrations, callose can be seen stained blue in visible light. Cellulose fluoresces pale blue after staining.

Berberine sulfate/crystal violet or aniline blue EX 400/EM 500 and above

0.1% berberine sulfate in water	alternative – 0.1% fluorol yellow 088
0.05% crystal violet in water	alternative – 0.05% aniline blue

Incubate fresh or resin sections in berberine sulfate for 30-60 min.
Transfer immediately to crystal violet solution, stain for 30-60 sec, or more if needed.
Rinse thoroughly in water before mounting in water for observation under blue light.

Fatty compounds and hydrophobic polymers like suberin and lignin fluoresce bright yellow, all remaining cell components are non-fluorescent or fluoresce deep red. Fluorol yellow 088 stains similarly to berberine sulfate. Crystal violet and aniline blue quench fluorescence from non-hydrophobic cell and tissue components.

Nile red EX 552/EM 636

Stock solution 100 mg/ml in DMSO.
Working solution, dilute 1/100 in water, vortex thoroughly and use immediately.
Observe under green light.

Nile red is a hydrophobic dye that binds selectively to neutral lipids. It is almost non-fluorescent in polar environments and becomes highly fluorescent in non-polar environments. It also binds to proteins.

Basic fuchsin EX 520/EM 570

0.1 – 1.0% basic fuchsin in water.

Basic fuchsin is the stain component in the Feulgen stain for DNA. It stains nuclei in acidified tissues bright red. It also stains bacteria and mucins.

Vital stains for microscopy

Vital dyes are used to distinguish living from dead cells. In general, a combination of two dyes is used. One dye is excluded from living cells – it cannot cross the cell membrane because it is too large or too highly charged to penetrate. However, this dye usually can stain dead plant tissues, cell walls and other material. The other dye, of contrasting colour or fluorescence, is taken up by the cell and accumulates in the cytoplasm or vacuole. Thus the cells are one colour if alive and a contrasting colour if dead. There is a large literature on various vital stains, as for other types of stains.

A common combination used for **brightfield microscopy** is Evans blue and Neutral red. Evans blue does not penetrate living cells, whereas neutral red accumulates in the cytoplasm and vacuole and stains living cells a pink-red colour.

Example protocol:
Stain cells or tissues with 0.1-0.5% Evans blue in aqueous solution. For delicate tissues, buffer to pH 6.5 and add appropriate osmoticum, e.g. 0.3 M mannitol.
Generally stain between 10 and 30 min, or longer for tissues with less permeable cell walls.
Rinse in water or buffer.
Stain with 0.01-0.1% Neutral red in aqueous solution. As with Evans blue staining, for delicate tissues, buffer to pH 6.5 and add appropriate osmoticum, e.g. 0.3 M mannitol.
As with Evans blue, generally stain between 10 and 30 min, or longer for tissues with less permeable cell walls.
Rinse and observe.

A common combination used for **fluorescence microscopy** is propidium iodide with fluorescein diacetate (FDA) or carboxyfluorescein diacetate (CFDA). Propidium iodide is non-fluorescent in aqueous solution and is charged, so cannot enter living cells with intact cell membranes. PI stains plant cell walls and cell nuclei as it is only fluorescent when it binds into certain polymer configurations, e.g between the base-pairs of DNA or between parallel strands of cellulose in plant cell walls. FDA and CFDA are also non-fluorescent in aqueous solution, and have neutral charge so they can cross the cell membrane and enter the cell. In the cytoplasm, the acetate groups are cleaved off the molecule by cytoplasmic esterases and the fluorescent molecules fluorescein or carboxyfluorescein accumulate within the living cell, often ending up in the vacuole. Neither of these fluorescent molecules are very membrane permeable, so they do not escape from the cell, at least in the short term. CF is less membrane-permeant than fluorescein, so less likely to leak out.

Example protocol:
Stain tissues with 0.001% (10μg/ml) propidium iodide and 0.0005% (0.5μg/ml) FDA or 0.001% (1μg/ml) CFDA. (If these concentrations do not produce significant fluorescence, increase the concentrations.) Tissues can be observed while in the staining solution. Under blue illumination, all cell walls, plus nuclei in dead cells, will fluoresce red, and cell contents in living cells will fluoresce bright yellow-green after 5-10 min. To stop uptake of the dyes, rinse tissues after 15 min. Fluorescein and carboxyfluorescein become toxic if too much of the dye accumulates in the cells, and PI is also slightly toxic and will eventually penetrate into living cells. The key is to observe soon after application of stain.
Stock solution of PI – 1 mg/ml in water stored in the fridge – lasts for a long time.
Stock solution of CFDA/FDA – 100 μg/ml in DMSO stored in fridge or freezer. Make up by adding correct amount to aqueous solution and vortexing immediately to get into solution.

Selected references:

Haseloff J (2003) Old botanical techniques for new microscopes. BioTechniques 34:1174-1182
Good outline of new ways to look at tissues treated with old clearing and staining techniques.

Lux A,Morita S, Abe J, Ito K (2005) An improved method for clearing and staining free-hand sections and whole-mount samples. Annals of Botany 96: 989-996
Nice paper, a number of staining protocols tested by the authors.

O'Brien TP, McCully ME (1981) The Study of Plant Structure: Principles and selected methods. Termarcarphi Pty Ltd.
A useful book, now long out of print, especially good for methods for looking at fresh tissues and for explaining what to expect. Infuriating lack of an index makes it hard to find your way around at first.

Ruzin SE (1999) Plant Microtechnique and Microscopy. Oxford University Press.
Covers the basics but deals mostly with fixed and/or resin-embedded tissues and sometimes doesn't give much detail on what to expect from the stains.

http://www.ffp.csiro.au/research/mycorrhiza/root.html - great web page by Mark Brundrett on staining roots, mainly tree roots and focussing on mycorrhizas, but the methods can be applied other roots and to other plant tissues.

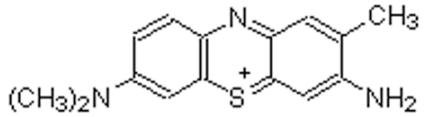
There are lots of web pages from various universities on preparing and staining freshly sectioned plant tissues, for example http://biology.unlv.edu/Schulte/BIO426/, http://www.plantsci.cam.ac.uk/Haseloff/ - which has a heap of other information on plant development and imaging, etc. etc.

A great web site about stains is http://stainsfile.info/StainsFile/ which has very detailed information about most stains for plant tissues.

Structure of dyes and stains

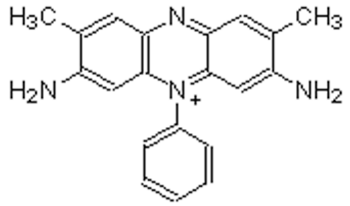
toluidine blue O

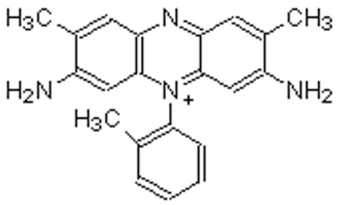
Molecular Formula: $C_{15}H_{16}N_3SCl$
Molecular Weight: 305.8



safranin

Molecular Formula: $C_{20}H_{19}N_4Cl$ (Dimethyl), $C_{21}H_{21}N_4Cl$ (Trimethyl)
Molecular Weight: 350.8 (Dimethyl), 364.9 (Trimethyl) 558.66



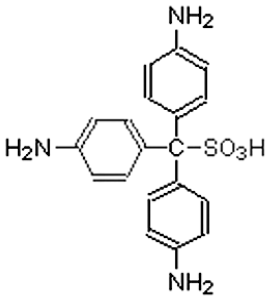


dimethyl safranin trimethyl safranin

Safranin O is a mixture of the two compounds shown according to Conn. Aldrich and Gurr give only the dimethyl compound.

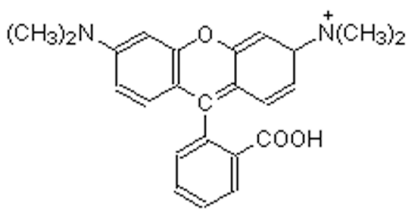
Schiff's reagent

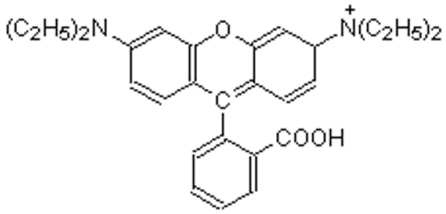
Molecular Formula: $C_{19}H_{19}SO_3N_3Cl$



rhodamine B

Molecular Formula: $C_{28}H_{31}N_2O_3Cl$
Molecular Weight: 479

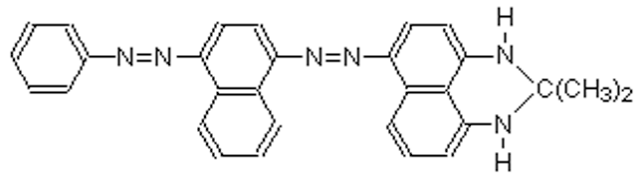




from Merck Index from Conn's Biological Stains

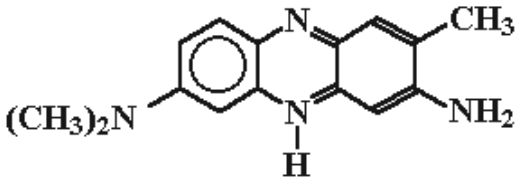
sudan black B

Molecular Formula: $C_{29}H_{24}N_6$
Molecular Weight: 456.6



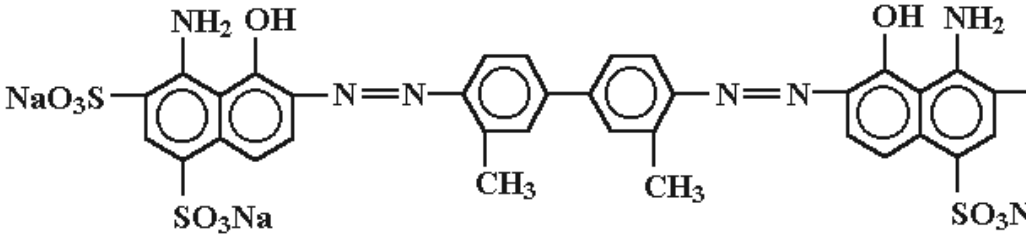
neutral red

Molecular Formula: $C_{15}H_{17}N_4Cl$
Molecular Weight: 288.78



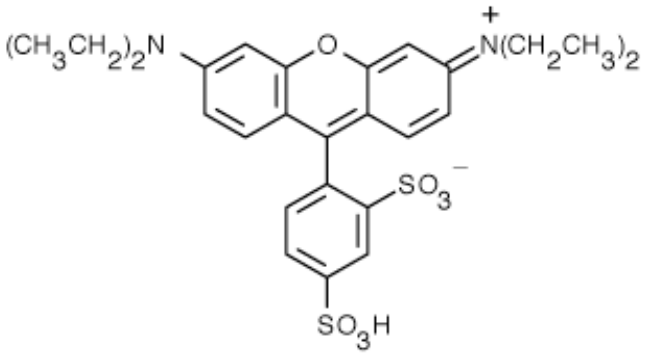
evans blue

Molecular formula: $C_{34}H_{24}N_6O_{14}S_4Na_4$
Molecular weight: 960.842



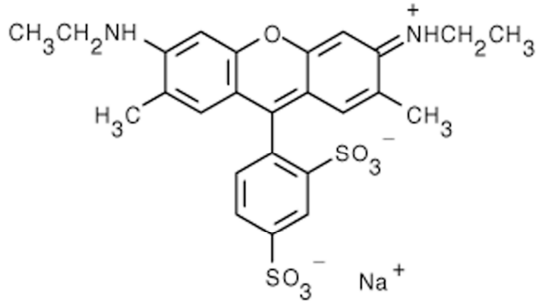
sulforhodamine B

Molecular Formula: $C_{27}H_{30}N_2O_7S_2$
Molecular Weight: 558.66
CAS Number/Name: 2609-88-3 / Xanthylium, 3,6-bis(diethylamino)-9-(2,4-disulfophenyl)-, inner salt



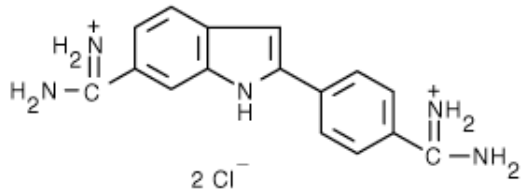
sulforhodamine G

Molecular Formula: $C_{25}H_{25}N_2NaO_7S_2$
Molecular Weight: 552.59
CAS Number/Name: 5873-16-5 / Xanthylium, 9-(2,4-disulfophenyl)-3,6-bis(ethylamino)-2,7-dimethyl-, inner salt, monosodium salt



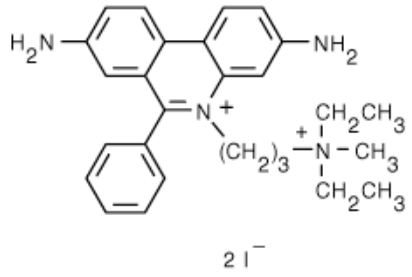
DAPI – 4',6-diamidino-2-phenylindole, dihydrochloride

Molecular Formula: $C_{16}H_{17}Cl_2N_5$
Molecular Weight: 350.25
CAS Number/Name: 28718-90-3 / 1H-Indole-6-carboximidamide, 2-[4-(aminoiminomethyl)phenyl]-, dihydrochloride



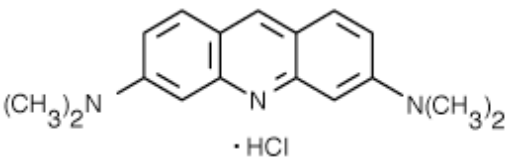
propidium iodide

Molecular Formula: $C_{27}H_{34}I_2N_4$
Molecular Weight: 668.40
CAS Number/Name: 25535-16-4 / Phenanthridinium, 3,8-diamino-5-[3-(diethylmethylammonio) propyl]-6-phenyl-, diiodide



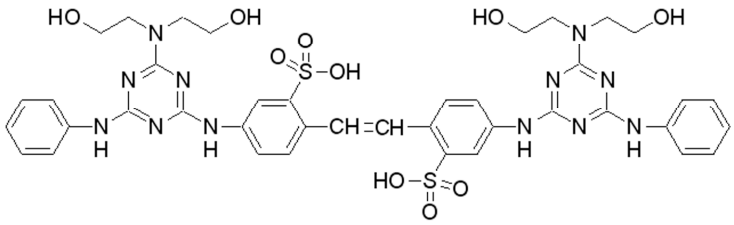
acridine orange

Molecular Formula: $C_{17}H_{20}ClN_3$
Molecular Weight: 301.82
CAS Number/Name: 65-61-2 / 3,6-Acridinediamine, N,N,N',N'-tetramethyl-, monohydrochloride



calcofluor white

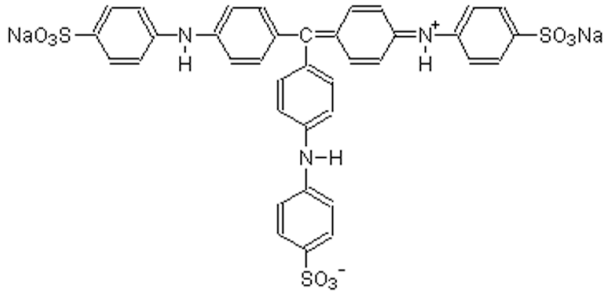
Molecular Formula: $C_{40}H_{44}N_{12}O_{10}S_2$
Molecular Weight: 916.98
CAS Number/Name: 4404-43-7 / 4,4'-bis((4-anilino-6-(bis(2-hydroxyethyl)amino)-S-triazin-2-yl)amino)-2,2'-stilbene disulfonic acid



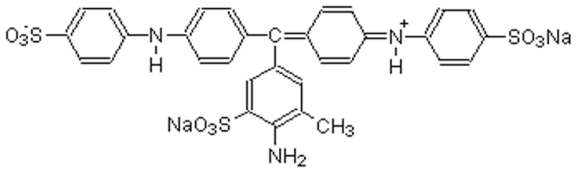
aniline blue

Aniline blue is a mixture of Methyl blue and Water blue or might be supplied as either one of them.

Methyl blue
Molecular Formula: $C_{37}H_{27}N_3O_9S_3Na_2$
Molecular Weight: 799.81

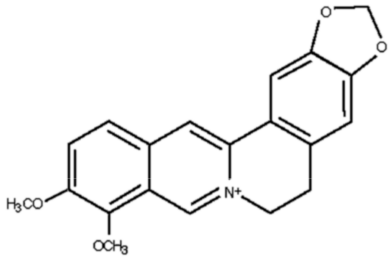


Water blue
$C_{32}H_{25}N_3O_9S_3Na_2$
737.756



berberine sulfate

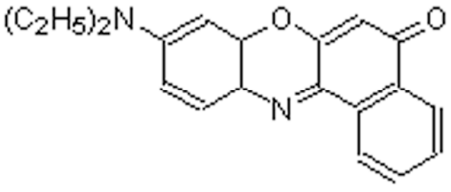
Molecular Formula: $C_{20}H_{24}NSO_{14}$
Molecular Weight: 438.4
5,6-Dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo(5,6-a)quinolizinium



+ $SO_4.3H_2O$

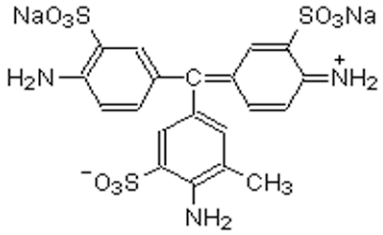
nile red

Molecular Formula: $C_{20}H_{18}N_2O_2$
Molecular Weight: 318.38



acid fuchsin

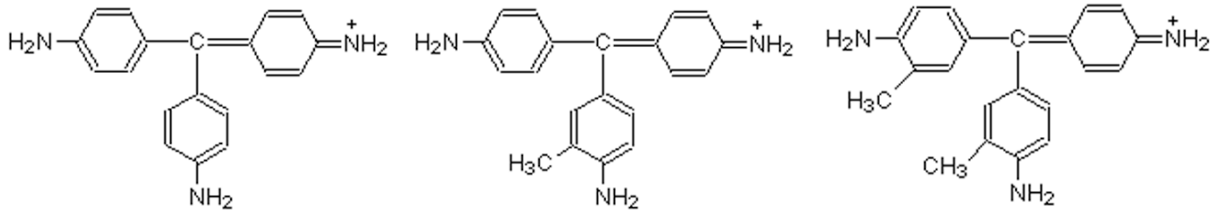
Molecular Formula: $C_{20}H_{17}N_3Na_2O_9S_3$
Molecular Weight: 585.55



basic fuchsin

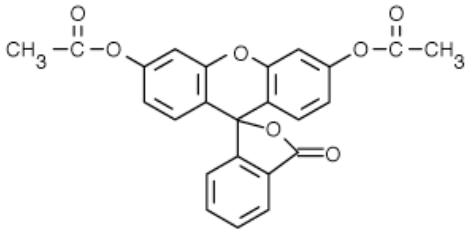
Basic fuchsin is a mixture of three chemically related dyes. However, the proportion of each dye in the mixture varies. The counter-ion is usually chloride.

	pararosanalin	rosanalin	magenta II
Molecular formula:	$C_{19}H_{18}N_3Cl$	$C_{20}H_{20}N_3Cl$	$C_{21}H_{22}N_3Cl$
Molecular weight:	323.834	337.861	351.888



FDA

Molecular Formula: $C_{24}H_{16}O_7$
Molecular Weight: 416.39
CAS Number/Name: 596-09-8 / Spiro(isobenzofuran-1(3H),9'-(9H)-xanthen) -3-one, 3',6'-bis(acetyloxy)-oxo-,(acetyloxy)methyl ester



CFDA

Molecular Formula: $C_{28}H_{20}O_{11}$
Molecular Weight: 532.46
CAS Number/Name: 124412-00-6 / Spiro(isobenzofuran-1(3H),9'-(9H)xanthene) -5-carboxylic acid, 3',6'-bis(acetyloxy)-oxo-,(acetyloxy)methyl ester