

# Fixatives

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This part is merely a selection of some major histological fixatives and not a compilation of all the formulas or schedules which have been developed in the history of histopathology. It is also not intended to consider tissue fixation in its complete extent. It will be pragmatic to mention standard fixatives for routine and fixatives for special purposes.

It is reminded that the properties of a fixative depend on both the physicochemical nature of the fixing agent and the vehicles used in the fixative solution. The efficiency of the different components will vary from tissue to tissue. Unfortunately, theoretical grounds are difficult to prove in complex biological situation (cells and tissues) or the outcome is difficult to predict under study conditions, fixation schedules are often a matter of trial and error. Apart from the physico-chemical behaviour of the fixing agent itself, other requirements must be followed:

- Maintenance of appropriate pH in the solution during the fixation process.
- Choice of suitable vehicles and appropriate osmolarity to avoid swelling or shrinkage as much as possible.
- Control of ionic and microenvironmental constitution within intracellular and extracellular spaces to minimize extraction and precipitation of macromolecules or cellular organelles.

Some general views on the fixation process of biological specimens are to be read in the chapter *Fixation of biological specimens*.

## Formaldehyde as fixative

The use of formalin as histological fixative dates back to the time when the synthesis of formaldehyde from methanol was successfully achieved by AW HOFMANN (1868). Since then, properties and potential use of this substance were intensively studied and formalin became attractive as an alternative reagent to the old established histological fixatives (LOEW O, 1886; BLUM F, 1893; CULLEN TS, 1895; BETHE A, 1896; BLUM F, 1896). Apart from tissue fixation, formaldehyde has become an important product for many applications, and its production reached large scales. Formaldehyde is sold as a saturated aqueous solution under the name *formalin* or *formaldehyde solution* (37-40%). Alternatively, formaldehyde can be obtained as a white crystalline product, i.e. *paraformaldehyde* which is polymerised formaldehyde.

Formalin is the most commonly used fixative in histopathology (FOX CH et al., 1985; KIERNAN JA, 2008). The solution should be stored at room temperature because cold temperature encourage the formation of trioxymethylene which gives a white precipitate. Furthermore, formaldehyde solutions should be stored tightly sealed in order to avoid exposure to air because oxidation of formaldehyde will lead to formic acid (commercial formalin usually contains some methanol to inhibit this change).

For historical reasons, there exists sometimes confusion about the difference between *formaldehyde* and *formalin* because both items are sometimes used interchangeably. However, it is incorrect to use the terms in this way since the concentrations of this chemical being represented by the two names are quite different.

Formaldehyde is a gas (formula HCHO). It dissolves readily into water which is called *formalin*. Formaldehyde dissolves into water to 37-40% (w/v), and this solution represents *100% formalin*. When diluting such a formalin solution, the final concentration is expressed based on the 100% formalin reference: 10% formalin is a 1:10 dilution of 100% formalin.

The terminology *10% formalin* is commonly used, i.e. a fixative labeled as **10% formalin** (buffered or not) contains actually **4% formaldehyde**. The expression 4% formaldehyde is also often used reflecting the actual formaldehyde content of a 10% formalin solution which describes a 10% solution made from a stock bottle of 37-40% formaldehyde (and, thus, giving a 3.7-4% solution of formaldehyde).

Formaldehyde dissolved in water combines chemically with its molecules to form methylene hydrate; it is in this form that formaldehyde exists in aqueous solutions. The chemical reactivity is the same as that of formaldehyde. Upon storage, methylene hydrate molecules react with one another forming polymers. Within the formalin stock solutions (37-40% formaldehyde in water) most of the formaldehyde will exist as low polymers. Such polymers will break up almost instantaneously by dilution with buffer at physiological pH.

Commercial formalin solution contain small amounts of methanol in order to slow down the polymerisation process during storage. Thus, a 10% formalin solution with 4% formaldehyde contains about 1% methanol and small amounts of formate ions, too. All these products other than formaldehyde are likely to interfere with histological processes. For this reason, formalin (prepared from a commercial 37-40% formaldehyde stock solutions) is not recommended as fixative for electron microscopy. It is proposed to use either a higher grade methanol-free solution or a freshly prepared formaldehyde made from formaldehyde powder (paraformaldehyde).

Paraformaldehyde is another form of formaldehyde. Formaldehyde can be polymerised to big molecules, and this product is called paraformaldehyde which can be obtained as a white powder. Depolymerisation of these polymers back to formaldehyde is done by heat; apart from heating, hydrolysis is further catalyzed by the addition of hydroxide ions (ROBERTSON JD et al., 1963).

## Glutaraldehyde

In the search for adequate fixatives for electron microscopy, major advances were made with the introduction of glutaraldehyde as primary fixative (SABATINI DD et al., 1963; SABATINI DD et al., 1964). The excellent preservation of fine structure by this dialdehyde has made glutaraldehyde fixation to become the standard in biological electron microscopy. This dialdehyde is more efficient than formaldehyde due to the rapid formation of intra- and intermolecular cross-links with tissue components. The mechanism of reaction is similar to that of formaldehyde, however, due to its two aldehyde groups and its greater molecular length glutaraldehyde is able to link faster and more distant pairs of protein molecules than

formaldehyde can. The degree of cross-linking depends mainly on the accessibility of  $\epsilon$ -amino groups leading to the formation of Schiff's bases.

For reproducible results, highly purified glutaraldehyde is needed. Glutaraldehyde may contain significant amounts of polymers, glutaric acid, inorganic substances etc. which all together can initiate unexpected reactions (especially important for cytochemical studies). The degree of purity of glutaraldehyde is expressed by the purification index (P.I.) where  $P.I = E_{235\text{ nm}} : E_{280\text{ nm}}$  and the P.I should be better than 0.2 for electron microscopic purposes as well as for other applications such as in histochemistry or in antibody and enzyme conjugation.

Glutaraldehyde of high purity are obtainable from commercial sources; stock solutions of 25% or higher can be purchased. Otherwise, the user can improve the degree of purity by easy means. The best way of purification is done by vacuum distillation using a Vigreux column. Glutaraldehyde stock solutions are preferably stored under refrigeration. For fixation purpose, glutaraldehyde is applied either as a single fixative or in combination with formaldehyde together with a suitable buffer system.

### **Other reagents for fixation**

There exist a number of other chemicals as protein cross-linking reagents which, however, are not widely employed. These include carbodiimides, diisocyanates, diazonium compounds, diimidoesters, diethylpyrocarbonate, maleimides, benzoquinones etc., just to mention some of the potential fixatives.

Other fixation agents such as *oxidising* or *precipitating agents* or *picrates* have also great potential as fixatives in cell preservation. Most of them have already been introduced about 100 years ago, i.e. in the era when histological fixation became a subject matter. Many of these fixatives are less common in immunohistology. Some formulations, however, are successfully modified for this purpose.

Due to the toxicity and the potential carcinogenicity of a number of chemicals, proprietary fixatives with reduced amounts of formalin or none at all (so-called formalin substitute fixatives) became developed by some companies. Their usefulness in histopathology is now under study (TITFORD ME and HORENSTEIN MG, 2005).

### **Selection of fixative solutions for histology**

Depending on the intended use, cells or tissues of different origin may need different fixation schedules. With respect to immunohistology, any kind of fixative can be imagined to be useful for immunostaining. The many fixation proposals published in the long history of microtechnique is hardly to summarize. Here, we can give only a selection of traditional and widely employed fixatives including some of the newer fixation formulations. Fixation solutions for the study of fine structural details ususally need more careful preparation than those for routine histopathological purpose. For the sake of morphology, it is often advised to compensate the tonicity of fixatives, f.e. with sodium chloride, calcium chloride, magnesium

chloride or sucrose. For detailed descriptions and formulas of biological fixatives with special reference to electron microscopy we refer to AM GLAUERT (1975) and G MILLONIG (1976).\*

## Acetone

Note: Acetone is not recommended as morphological fixative for tissue blocks, mainly because of its shrinkage and poor preservation effects. Its use is reserved for the fixation of cryostat sections or for tissues in which enzymes have to be preserved. Acetone is almost used alone and without dilution, it fixes by dehydration and precipitation

Acetone, pure and water free	-	-
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Fixation: Acetone is used to fix specimens at cold temperatures (0 to 4°C). Fixation time may vary from several minutes (for cell smears, cryostat sections) to several hours (1-24 hours for small tissue blocks)

Subsequent: Acetone is a dehydrant, thus no special treatment is needed after fixation. Specimens can be directly transferred into hot paraffin for embedment

## Bouin fixative

Note: 1.2% picric acid in distilled water corresponds to a saturated picric acid solution in water

Picric acid solution (1% in dist. water)	15.0	mL
Formalin stock solution (40%)	5.0	mL
Glacial acetic acid	1.0	mL

Fixation: Fixative is prepared immediately prior to use. Specimens are fixed for up to 24 hours

Subsequent: Specimens are washed in 50% to 70% ethanol to remove picric acid (yellow color). Then, the tissue can be stored in 70% ethanol for a longer time. When washing is done in water, add a few drops of saturated lithium carbonate until the color is removed

## Bouin fixative, alcoholic modification (Duboscq, Brasil)

Stock solution A: 1.0 g picric acid dissolved in	Alcoholic Bouin's solution:
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\* Fixatives and buffer systems can be toxic. They must be handled with great care. Be aware, some fixative solutions contain mercury salts. Do not contaminate wastewater. Follow legal regulations for disposal of waste and chemicals

150.0 mL 80% ethanol in water, then add 60.0 mL formalin stock solution (40%)	the solution is stable for 1 year
Solution B: glacial acetic acid	
Preparation of fixative (working solution is prepared immediately prior to use: 70.0 mL of alcoholic Bouin's solution (stock A) is mixed with 5.0 mL glacial acetic acid (solution B)	

Fixation: Tissue specimens such as biopsies are fixed for 1 to several hours, then directly transferred into 90-95% ethanol (several changes), followed by absolute ethanol for dehydration

### **Bouin fixative, cupric acetate modification (Hollande)**

Picric acid	40.0	g
Cupric acetate	25.0	g
Formalin stock solution (40%)	100.0	mL
Glacial acetic acid	15.0	mL
Distilled water	1000	mL
Dissolve copper acetate in distilled water, add picric acid slowly and stir until dissolved; add formaldehyde and acetic acid. This fixative is a modification of Bouin's fixative. The solution is stable for 1 year		

Fixation: Tissues are fixed for up to 24 hours. Do not allow Hollande fixed specimens to come into contact with phosphate buffered formaldehyde (precipitates, the fixative must be washed out before)

Subsequent: Specimens are washed in distilled water, then in 50 to 70% ethanol to remove picric acid (tissues can be stored in 70% ethanol for a longer time) and followed by ascending series of ethanol for dehydration

### **Carnoy fixative (van Gehuchten's fluid)**

Absolute ethanol	60.0	mL
Chloroform	30.0	mL
Glacial acetic acid	10.0	mL

Fixation: Tissues are fixed for 1-12 hours depending on the thickness of specimens, e.g. 1 hour for 1-2 mm thick tissue blocks

Subsequent: Tissues are transferred into absolute ethanol (for 24 hours) which is changed several times and followed by embedment

### **Carnoy fixative, methanol modification (*Methacarn* by Puchtler et al.)**

Note: Methacarn is Carnoy's fluid in which ethanol is replaced by methanol. Morphological preservation is described to be superior to Carnoy's fixation. Also, it seems to preserve antigens more efficiently than Carnoy's solution. Methacarn has been recommended for DNA and RNA studies

Methanol, absolute	60.0	mL
Chloroform	30.0	mL
Glacial acetic acid	10.0	mL

Fixation: Tissues are fixed for 1-12 hours depending on the thickness of specimens, e.g. 1 hour for 1-2 mm thick tissue blocks. Yet, fixation can be prolonged for up to 24 hours with little harm

Subsequent: Tissues are transferred into absolute ethanol, frequently changed (usually for 24 hours as long as one smells acetic acid) which is followed by embedment

### **Ethanol fixative (Sainte-Marie)**

Ethanol (absolute)	95.0	mL
Distilled water	5.0	mL

Fixation: Tissue blocks (about 2-4 mm thick) are fixed for 24 hours at 2-6°C

Subsequent: Specimens are transferred into pre-cooled absolute ethanol at 4°C and dehydrated in four changes, 1 to 2 hours each; agitation will accelerate dehydration. The, tissue blocks are cleared by passing through three consecutive baths of pre-cooled xylene for 1 to 2 hours at 4°C. Vessels of the last xylene bath are allowed to come to room temperature. Specimens are embedded in paraffin at 56°C

### **Ethanol-acetic acid fixative (Clarke)**

Ethanol (99%)	75.0	mL
Glacial acetic acid	25.0	mL

Fixation: Tissue blocks (about 2-5 mm thick) are fixed in ethanol pre-cooled to 4°C for a total of 15-24 hours

Subsequent: Specimens are transferred into 70% ethanol at 2-6°C (where they can be also stored), followed by ascending series of ethanol for dehydration

### **Ethanol-acetic acid fixative (Kuhlmann, Kuhlmann and Peschke)**

Note: For a great variety of tissues, a modification of the original Clarke's fixation proved very useful in the immunohistochemical study of antigens. Due to its poor penetration, ethanol is not widely used in histology. However, small tissue blocks or (even better) tissue slices of about 2-4 mm thickness are surprisingly well fixed by this fixative. In routine, we use a mixture of 99 parts of absolute ethanol plus 1 part of glacial acetic acid, thus giving a fixative solution with a final concentration of 99% ethanol and 1% acetic acid. It was found that sections of tissue blocks from many organs fixed in this way are well suited for routine histology with classical histologic stainings as well as for immunoenzyme labelings.

Tissues fixed with solutions composed of variable amounts of ethanol and acetic acid and which are embedded in paraffin embedment have also proved useful in immunofluorescent studies

Ethanol (absolute)	96.0-99	mL
Glacial acetic acid	1.0-4.0	mL

Fixation: Tissue slices (about 2-4 mm thick) are fixed for 24 hours at 0-6°C under continuous agitation with several changes of the fixative

Subsequent: Specimens are transferred into absolute ethanol at 2-6°C for about 2 hours with two changes of absolute ethanol, then brought to room temperature and followed by at least 2 changes of absolute ethanol for dehydration. Best results are obtained with fixation and dehydration under continuous agitation

### **Formaldehyde fixative**

Formalin stock solution (40%)	10.0	mL
Distilled water	90.0	mL

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens, e.g. 6 hours for small tissue blocks; longer fixation is not harmful. Fixation can be accelerated at higher temperatures (f.e. 40-50°C)

Subsequent: Tissues are carefully washed in water, then dehydrated in ascending series of ethanol (70%, 96%, absolute ethanol) and embedded

### **Formaldehyde fixative (buffered formalin)**

Formalin stock solution (40%)	10.0	mL
0.2 M phosphate buffer pH 7.2 or pH 7.4	90.0	mL

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens, e.g. 6 hours for small tissue blocks; longer fixation is not harmful

Subsequent: Tissues are washed in phosphate buffered solution, then dehydrated in ascending series of ethanol (70%, 96%, absolute ethanol) and embedded

### **Formaldehyde fixative (buffered formalin freshly prepared from paraformaldehyde)**

Paraformaldehyde (powder)	8.0	g
0.2 M sodium cacodylate buffer pH 7.2.-7.4	100.0	mL

Solution: For the preparation of 200 mL of 4% formaldehyde solution, add 8 g paraformaldehyde powder to 100 mL distilled water and heat in a fume hood to 60°C. Add dropwise 1 M sodium hydroxide to dissolve paraformaldehyde; paraformaldehyde depolymerizes to give formaldehyde, and the resulting gas dissolves immediately making a formalin solution (the solution will become clear). Cool under the faucet, filter and add 100 mL cacodylate buffer

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens, e.g. 6 hours for small tissue blocks; longer fixation is not harmful

Subsequent: Tissues are transferred into phosphate buffer solution and washed as long as one smells formaldehyde

### **Formalin-acetic acid-zinc chloride fixative (acetic acid-zink-formalin, AZF)**

Note: This fixative is recommended as replacement for B5 fixative (mercury based fixative)

Formalin stock solution (40%)	30.0	mL
Glacial acetic acid	2.0	mL
Zinc chloride	5.0	g

Distilled water added to give	200.0	mL
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Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens, e.g. 6 hours for small tissue blocks; longer fixation is not harmful

Subsequent: Specimens are transferred into water with several washings, followed by 70% ethanol and ascending series of ethanol for dehydration

### **Formalin-ethanol fixative (Schaffer)**

Formalin stock solution (40%)	10.0	mL
Ethanol (80-96%)	20.0	mL

Fixation: Tissues are fixed for up to 1-2 days depending on the thickness of specimens; longer fixation is usually not harmful

Subsequent: Tissues are transferred into 80% ethanol and washed in several changes of 80% ethanol; then 96% ethanol and absolute ethanol for dehydration, followed by embedment

### **Formalin-ethanol-acetic acid fixative (Scheuring)**

Formalin stock solution (40%)	48.0	mL
Ethanol (absolute)	48.0	mL
Glacial acetic acid	4.0	mL

Fixation: Tissues are fixed for up to 1-2 days depending on the thickness of specimens

Subsequent: Tissue blocks are transferred into 80% ethanol and washed (until one no longer smells formaldehyde) followed by absolute ethanol for dehydration

### **Formalin-ethanol-acetic acid fixative (Lavdowsky)**

Note: Fixative used for fixation of nuclei in botanical and animal specimens

Formalin stock solution (40%)	30.0	mL
Ethanol (95%)	100.0	mL
Glacial acetic acid	5.0	mL

Distilled water	200.0	mL
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Fixation: Tissues are fixed for up to 1-2 days depending on the thickness of specimens

Subsequent: Tissue blocks are transferred into 80% ethanol and washed (until one no longer smells formaldehyde) followed by absolute ethanol for dehydration

### **Formalin-ethanol-acetic acid fixative (Davidson's or Hartmann's fixative, reported by Moore KL et al.)**

Note: This fixative can be used for fixation of histological specimens or to preserve samples for in-situ-hybridization

Formalin stock solution (40%)	80.0	mL
Ethanol (95%)	120.0	mL
Glacial acetic acid	40.0	mL
Distilled water	120.0	mL

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens

Subsequent: Specimens are transferred into 70% ethanol and washed (until one no longer smells formaldehyde and acetic acid). Tissues are dehydrated by ascending series of ethanol

### **Formaldehyde-periodate-lysine fixative (McLean and Nakane)**

Solution A: 0.1 M lysine in 0.1 M phosphate buffer pH 7.4	
Solution B: 8% formaldehyde (from paraformaldehyde)	
Working solution: 3 parts of solution A mixed with 1 part of solution B and add NaIO <sub>4</sub> to 0.01 M (= 21.4 mg/10 mL)	Final concentrations: 2% formaldehyde 0.075 M lysine 0.01 M NaIO <sub>4</sub> 0.0375 phosphate buffer
Preparation of fixative: (a) Lysine solution (A): 1.827 g L-lysine-HCl dissolved in 50.0 mL distilled water adjust pH to 7.4 with 0.1 M Na <sub>2</sub> HPO <sub>4</sub> and add	

0.1 M phosphate buffer pH 7.4 to give a final volume of 100.0 mL.  
The solution can be stored at 4°C

- (b) Formaldehyde solution (B):  
8 g paraformaldehyde powder are mixed in 100.0 mL distilled water, heat in a fume hood to 60°C with stirring and add 1 M NaOH (dropwise) to dissolve paraformaldehyde; paraformaldehyde depolymerizes to give formaldehyde, and the solution will become clear. Cool under the faucet, filter and store at 4°C
- (b) Working solution *for immediate use*:  
3 parts of solution A are mixed with 1 part of solution B, then add sodium *m*-periodate (NaIO<sub>4</sub>, 21.4 mg per 10 mL fixative) which gives 0.01 M periodate, finally

Note: the pH will decrease upon the addition of NaIO<sub>4</sub>, but the tissues are fixed without readjusting the pH

Fixation: For electron microscopy, tissue blocks (2-4 mm pieces) are fixed for 3 hours at 4°C, followed by postfixation in 1% osmium tetroxide in phosphate buffer for 1 hour

Subsequent: Specimens are dehydrated in ascending series of ethanol and embedded

### Formalin-potassium bichromate-acetic acid fixative (Ciaccio)

Potassium bichromate (5% solution)	74.7	mL
Formalin stock solution (40%)	20.6	mL
Glacial acetic acid	4.7	mL

Fixation: Tissues are fixed for up to 48 hours depending on the thickness of specimens

Subsequent: Specimens are transferred into water and washed for 24 hours (tap water). Dehydration is done by ascending series of ethanol

### Formalin-zinc chloride fixative

Formalin stock solution (40%)	10.0	mL
Zinc chloride	1.0	g
Distilled water (or selected buffers)	90.0	mL

Fixation: Tissues are fixed for 4-12 hours

Subsequent: Tissues are transferred into 70% ethanol (several changes of the ethanol solution), followed by dehydration in ascending series of ethanol

## Glutaraldehyde fixative

Note: This fixative is mainly used for electron microscopic purposes. The formula of a 1% glutaraldehyde fixative is given, and fixatives up to 3% glutaraldehyde are quite common. Glutaraldehyde can be well combined with formaldehyde

Glutaraldehyde solution (25%), preferentially of high purity	40	mL
Sodium dihydrogen phosphate (NaH <sub>2</sub> PO <sub>4</sub> )	2.98	g
disodium hydrogen phosphate (Na <sub>2</sub> HPO <sub>4</sub> )	30.40	g
Distilled water ad 1000 mL		

Fixation: Small tissues of about 1-2 mm<sup>3</sup> are fixed for 1-6 hours; specimens can be stored in fixative for longer times

Subsequent: Tissues are washed in several changes of phosphate buffer solution followed by dehydration and embedment

## Glutaraldehyde-formaldehyde fixative (Karnovsky)

Note: This fixative is mainly used for electron microscopic purposes. The formula of a fixative containing 0.25% glutaraldehyde and 2% formaldehyde is given. Fixative concentration can be varied according to the material to be studied by changing the respective volumes of glutaraldehyde, formaldehyde and buffer

Glutaraldehyde solution (25%), preferentially of high purity	1.0	mL
Formaldehyde (8%) freshly prepared from paraformaldehyde	25.0	mL
0.2 M phosphate buffer pH 7.2-7.4	74.0	mL

Solution: For the preparation of 100 mL of 8% formaldehyde solution, add 8 g paraformaldehyde to 100 mL distilled water and heat in a fume hood to 60°C. Add dropwise 1 M sodium hydroxide to dissolve paraformaldehyde (the solution will become clear). Cool under the faucet and filter

Fixation: Small tissues of about 1-2 mm<sup>3</sup> are fixed for 1-6 hours; specimens can be stored in fixative for longer times

Subsequent: Tissues are washed in several changes of phosphate buffer solution; dehydration in ascending ethanol series and followed by embedment

## Methanol

Note: Most common fixative for blood and bone marrow smears in connection with Romanovsky stainings. Methanol is rarely used alone as fixative for tissue blocks, but it is a very good substitute for ethanol in Carnoy's fluid (which is called Methacarn). Furthermore, methanol can be applied to cryostat sections. Methanol fixes by dehydration and precipitation

Methanol, pure and water free	-	-
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Fixation: It is used to fix specimens at cold temperatures (0 to 4°C). Fixation time may vary from several minutes (cell smears, cryostat sections) to 1-24 hours (small tissue blocks)

Subsequent: Methanol is a dehydrant, thus no particular treatment is needed. Tissue blocks are directly transferred into a clearing agent (or to ensure dehydration, specimens are passed into absolute ethanol) followed by hot paraffin for embedment

## Müller fixative

Potassium bichromate	2.5	g
Sodium sulfate (cris.)	1.0	g
Distilled water ad 100 mL		

Fixation: Tissue blocks (about 2 to 4 mm thick) are fixed for 24-48 hours, preferentially in the dark

Subsequent: Specimens are washed in running tap water, followed by ascending series of ethanol

## Orth fixative

Müller's solution (i.e. Müller fixative)	90.0	mL
Formalin stock solution (40%)	10.0	mL

Fixation: Tissue blocks (about 2 to 4 mm thick) are fixed for 24-48 hours, preferentially in the dark

Subsequent: Specimens are washed in running tap water, followed by ascending series of ethanol

### **Osmium tetroxide fixative (Schultze, Rudneff)**

Note: Fixative proposed for light microscopy. This fixative is applied for certain studies in the light microscope. Today, osmium tetroxide is mainly used in electron microscopy.

Osmium tetroxide is quite toxic and must be used in a fume hood; ensure that the fumes are not inhaled. Osmium is usually purchased in sealed glass vials; make a solution at an appropriate concentration (store well capped in the dark), then add the required volume to any other reagent (f.e. phosphate buffer) to prepare the fixative.

Osmium tetroxide is compatible with many other solutions (with the exception of ethanol and formaldehyde), and combinations of osmium with chromic acid, acetic acid, potassium bichromate etc. have been published

2% Osmium tetroxide in distilled water (osmium tetroxide is obtained in sealed ampoules; crystallized or as aqueous solution)	2.0	mL
Distilled water (or 0.1% chromic acid)	2.0	mL

Fixation: Small tissue blocks (1-2 mm) are fixed in osmium tetroxide for several hours (up to 24 hours and longer) at room temperature, then washed very well with running tap water (for several hours); tissue blocks are then washed in several changes of 70% ethanol; dehydration by ascending series of ethanol and followed by embedment

### **Osmium tetroxide fixative (Flemming)**

Note: Fixative for use in light microscopy; osmium tetroxide fixation with minor modification of Flemming's fluid was proposed f.e. by C Benda (1903) and M Lavdowsky (1894). Due to the different diffusion rates of the fixative components, the fixation effect differs somewhat within the specimens

2% Osmium tetroxide in distilled water (osmium tetroxide is obtained in sealed ampoules; crystallized or as aqueous solution)	20.0	mL
1% Chromium (VI) oxide in distilled water	75.0	mL
Glacial acetic acid	5.0	mL

Fixation: Small tissue blocks (1-2 mm) are fixed in osmium tetroxide for several hours (up to 24 hours or longer) at room temperature, then washed very well with running tap water (for several hours); tissue blocks are then passed into ascending series of ethanol (40%, 70%, 90%, absolute ethanol, each step for several hours) followed by embedment

### Osmium tetroxide fixative (Hermann)

Note: Fixative for use in light microscopy

2% Osmium tetroxide in distilled water (osmium tetroxide is obtained in sealed ampoules; crystallized or as aqueous solution)	20.0	mL
1% Platinum (IV) chloride in distilled water	75.0	mL
Glacial acetic acid	5.0	mL

Fixation: Small tissue blocks (1-2 mm) are fixed in osmium tetroxide for several hours (up to 24 hours and longer) at room temperature, then washed very well with running tap water (for several hours); tissue blocks are then washed in several changes of 70% ethanol; dehydration by ascending series of ethanol and followed by embedment

### Osmium tetroxide fixative (vom Rath)

Note: Fixative for use in light microscopy (picric acid-osmium-acetic acid fixative)

Picric acid solution, ca. 1.2% (saturated picric acid in distilled water)	93.46	mL
2% Osmium tetroxide in distilled water (osmium tetroxide is obtained in sealed ampoules; crystallized or as aqueous solution)	5.61	mL
Glacial acetic acid	0.93	mL
Preparation of saturated picric acid in distilled water: 3.0 g of picric acid are mixed with 200.0 mL hot distilled water, shake several times and let cool down over night to room temperature; the saturated solution will always show some precipitates		

Fixation: Small tissue blocks (1-2 mm) are fixed for several hours (up to 24 hours), then washed in 70-75% ethanol containing some lithium carbonate (several

hours); tissue blocks are dehydrated by ascending series of ethanol and followed by embedment

### Osmium tetroxide fixative (Millonig)

Note: Fixative for electron microscopic specimens. Osmium tetroxide is used either as a primary fixative or (mainly) as a secondary fixative (postfixation) which follows previous fixation in aldehydes).

Furthermore, osmium tetroxide is useful in diaminobenzidine cytochemistry for color enhancement of oxidized diaminobenzidine (histochemistry, immunohistochemistry, immunoperoxidase staining)

2% Osmium tetroxide in distilled water (osmium tetroxide obtained in sealed ampoules either crystallized or as aqueous solution)	2.0	mL
0.1 M phosphate buffer pH 7.2-7.4 For tonicity, CaCl <sub>2</sub> or MgCl <sub>2</sub> can be added if required. Avoid the formation of precipitates, thus, tests for precipitation should be run at the temperature used for fixation  Note: other buffer systems such as 0.1 or 0.2 M cacodylate buffer pH 7.2-7.4 are also useful, f.e. when the primary fixation has been done with aldehyde in cacodylate buffer	2.0	mL

Fixation: Tissue blocks are fixed in buffered osmium tetroxide for 1-2 hours at room temperature, then washed in several changes of buffer, followed by 70% ethanol (several changes) and ascending series of ethanol for dehydration and embedment.

In the case of color enhancement of immunoperoxidase stained histological sections (light microscopy), preparations are just covered with osmium tetroxide solution for 1-2 min at room temperature (work under hood). Then, sections are washed with distilled water, followed by several changes of 70% ethanol. Sections are dehydrated in ethanol and mounter under coverglass

### Osmium tetroxide fixative (Palade)

Note: Fixative for electron microscopic specimens. Several modifications of Palade's method have been published (Rhodin J, 1954; Zetterquist H, 1956; Caulfield JB, 1957) with suggestions to add salts or sucrose to adjust osmolarity

Solution A: 2% Osmium tetroxide in distilled water (osmium tetroxide obtained in sealed ampoules either crystallized or as aqueous solution)	
Solution B: Veronal-acetate stock solution (see chapter <i>Buffer solutions</i> )	Preparation of solution B: 2.95 g sodium veronal <i>plus</i> 1.94 g sodium acetate · 3 H <sub>2</sub> O are dissolved in distilled water to make 100.0 mL
Solution C: 0.1 M HCl in distilled water	
Solution D: Distilled water	
Preparation of fixative (the final solution is stable for at least 1 week at room temperature):  12.5 mL of osmium tetroxide solution (A) is mixed with 5.0 mL veronal-acetate stock solution (B) and add approx. 5.0 mL of 0.1 M HCl solution (C) to adjust pH (usually 7.2.-7.4), add distilled water to make a final volume of 25.0 mL	

Fixation: Tissue blocks are fixed in buffered osmium tetroxide for 1-2 hours at room temperature, then washed in several changes of buffer, followed by 70% (several changes) and ascending series of ethanol for dehydration and embedment

### **Osmium tetroxide-collidine buffered fixative (Bennett and Luft)**

Note: Fixative for electron microscopic specimens

Solution A: 2% Osmium tetroxide in distilled water (osmium tetroxide obtained in sealed ampoules either crystallized or as aqueous solution)	
Solution B: s-collidine buffer (see chapter <i>Buffer solutions</i> )	

Preparation of fixative (the final solution is stable for several days):

2.0 mL of osmium tetroxide solution (A) is mixed with  
1.0 mL *s*-collidine buffer (B)

Note: the final concentration of osmium tetroxide is 1.33%; for other OsO<sub>4</sub>-  
concentration use varying ratios of the two solutions

Fixation: Tissue blocks are fixed for about 1 hour at room temperature, then washed  
in collidine buffer and in several changes of 70% ethanol followed by  
ascending series of ethanol for dehydration and embedment

### Osmium tetroxide-potassium dichromate fixative (Dalton)

Note: Fixative for electron microscopic specimens

Solution A: 2.0 g potassium dichromate dissolved in 40.0 mL distilled water, then add 5 M NaOH to adjust pH to 7.2	
Solution B: 3.4% NaCl in distilled water	
Solution C: 2% osmium tetroxide in distilled water	
Preparation of fixative (the final solution is stable for several months at 4-6°C): 4.0 mL of potassium dichromate solution (A) is mixed with 4.0 mL sodium chloride stock solution (B) and add 8.0 mL osmium tetroxide solution (C)	

Fixation: Tissue blocks are fixed for 1 to 24 hours, then washed in several changes  
of 0.9% sodium chloride, followed by 70% (several changes) and ascending  
series of ethanol for dehydration and embedment

### Picric acid-ethanol-formalin-acetic acid fixative (Lison, Vokaer)

Saturated picric acid in ethanol	85.0	mL
Formalin stock solution (40%)	10.0	mL
Glacial acetic acid	5.0	mL

Fixation: Tissue blocks (about 2 to 4 mm thick) are fixed in the cold (4°C) for 6 to 12 hours; specimens can be stored in fixative for up to 24 hours

Subsequent: Specimens are washed and dehydrated in absolute ethanol

### **Picric acid-formaldehyde fixative (Zamboni)**

Saturated picric acid in water (ca. 1.2%)	15.0	mL
Paraformaldehyde powder	2.0	g
Phosphate buffer pH 7.3 (Sörensen)	ad 100.0	mL

Solution: For the preparation of 500 mL solution, add 10 g of paraformaldehyde to 75 mL of saturated picric acid and warm up to 60°C. The mixture is neutralized by dropwise addition of 2.5% sodium hydroxide (the solution will become clear). The mixture is filtered and, finally, phosphate buffer is added to give a volume of 500 mL. The fixative can be used for light and electron microscopic studies

Fixation: Tissue blocks (about 2 to 4 mm thick) are fixed for at least 24 hours. Specimens are washed in 70% ethanol, followed by dehydration in ascending series of ethanol

### **Picric acid-formaldehyde-glutaraldehyde fixative (Ito and Karnovsky)**

Note: Fixation can be used for light and electron microscopic specimens (see also Zamboni fixative)

Stock solution A: saturated picric acid in water (ca. 1.2%)	
Stock solution B: 10% formaldehyde solution, freshly prepared from paraformaldehyde powder	
Solution C: 0.2 M sodium cacodylate buffer pH 7.2-7.4	
Solution D: 25% glutaraldehyde (high purity)	
Solution E: 5% calcium chloride	

Working solution:	Final concentrations: 2.5 % formaldehyde 2.5 % glutaraldehyde 0.06 % picric acid 0.08 % calcium chloride 0.01 M cacodylate buffer
Preparation of solutions:	
(a) Stock A (saturated picric acid): solid picric acid is added to distilled water until the yellow solution becomes saturated (visible precipitates, 1.3% picric acid)	
(b) Stock B (formaldehyde solution): 10 g paraformaldehyde powder are mixed in 100 mL distilled water, heat in a fume hood to 60°C with stirring and add 1 M NaOH (dropwise) to dissolve paraformaldehyde	
(c) Working solution <i>for immediate use</i> : 4.6 mL stock A are mixed with 25.0 mL stock B (while still warm) add 50.0 mL solution C (cacodylate buffer) add 10.0 mL solution D (glutaraldehyde) add 1.6 mL solution E (calcium chloride) and add distilled water to give 100.0 mL, finally	

Fixation: Small tissue blocks are immersed in fixative for 2 hours

Subsequent: Specimens are rinsed several times with cacodylate buffer until the solution runs clear (one to several hours); postfixation with osmium tetroxide, dehydration in ascending series of ethanol followed by embedment

### Potassium bichromate-formalin fixative (Kopsch-Regaud)

Note: Fixative for light microscopic specimens

Potassium bichromate, 3%	80.0	mL
Formalin stock solution (40%)	20.0	mL

Fixation: Fixative is prepared immediately prior to use by. Fixation time is about 4 days. The fixative solution is changed every day. Then, specimens are treated with 3% potassium bichromate for 8 days

Subsequent: Tissues are washed in water for 24 hours, followed by ascending series of ethanol (70% ethanol, 90% ethanol and, finally, absolute ethanol)

### Potassium permanganate fixative (Luft)

Note: Fixative not suitable as a general fixative for electron microscopy (extraction of many cell components); useful for botanical studies

Solution A: 1.44g veronal sodium and 0.95g sodium acetate · 3 H <sub>2</sub> O dissolved in 50.0 mL distilled water	
Solution B: 0.6 g potassium permanganate dissolved in 50.0 mL distilled water	
Solution C: 0.1 M HCl in distilled water	
Preparation of fixative (this solution is prepared freshly prior to use): 5.0 mL of veronal-acetate solution (A) is mixed with 12.5 mL potassium permanganate solution (B), then add approx. 5.0 mL HCl solution (C) to adjust pH to 7.2-7.6	

Fixation: Specimens are fixed for 1 hour at room temperature, then carefully rinsed in several changes of sodium chloride (0.5-2% NaCl in distilled water) followed by ethanol dehydration and embedment

## Sublimate fixatives

General remarks: sublimate (mercury chloride, *hydrargyrum bichloratum*) was introduced by A LANG (1878) for fixation purposes and has become known as LANG's fluid. Mercuric chloride is one of the most effective fixatives. It is applied in aqueous solution; concentrations up to 6% are close to the saturation point in water. However, when used alone as fixative, sublimate will cause considerable cytoplasmic shrinkage. Thus, several modifications of sublimate fixatives which contain ethanol, acetic acid, formalin, potassium bichromate etc. were proposed (f.e. HEIDENHAIN, HELLY, STIEVE, ZENKER, ZENKER-formol fixatives). Such sublimate mixtures could overcome many of the artefactual phenomena and became very popular in histopathology.

Note: Mercuric chloride is very poisonous. Due to its toxicity and its ability to enter the food chain, it must be always handled with great care. Repeated exposures can accumulate over the time to produce serious side effects.

## Sublimate fixative (Lang)

Mercuric chloride (HgCl <sub>2</sub> )	5.0	g
Sodium chloride	6.0	g

Glacial acetic acid	5.0	mL
Distilled water ad 100.0 mL		

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens

Subsequent: Tissues are washed in several changes of 70% ethanol, passed through graded ethanol for dehydration. For the elimination of mercury precipitates see chapter “*Elimination of mercury salts from tissue sections*” or as stated below at *Zenker fixative*

### Sublimate-acetate fixative (B5 fixative, Lillie and Fullmer)

B5 stock solution: Mercuric chloride plus Sodium acetate plus Distilled water  Be aware: mercuric chloride reacts with metals, and all metal should be avoided when preparing or using mercuric chloride solutions	12.0 2.5 200.0	g g mL
Formalin stock solution (40%)	-	-
B5 working solution: B5 stock solution is mixed with Formalin stock solution	20.0 2.0	mL mL

Fixation: Fixative is prepared immediately prior to use; filter if not clear. Fixation time may vary from 30 minutes to up to 24 hours (or longer) depending on the thickness of the specimens

Subsequent: Tissues are washed in several changes of 70% ethanol, followed by ascending series of ethanol for dehydration (for the elimination of mercury precipitates see “*Elimination of mercury salts from tissue sections*” or as stated below *Zenker fixative*)

### Sublimate-formalin fixative (Heidenhain)

Formalin stock solution (40%)	20.0	mL
Sublimate (HgCl <sub>2</sub> )	4.5	g
Sodium chloride	0.5	g
Distilled water	80.0	mL

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens

Subsequent: Tissues are transferred into 70% ethanol and then passed through graded ethanol for dehydration (for the elimination of mercury precipitates see chapter "*Elimination of mercury salts from tissue sections*" or as stated below at *Zenker fixative*)

### **Sublimate-salt (*SuSa*)-formalin fixative (Heidenhain)**

Formalin stock solution (40%)	20.0	mL
Sublimate (HgCl <sub>2</sub> )	4.5	g
Sodium chloride	0.5	g
Trichloroacetic acid	2.0	g
Glacial acetic acid	4.0	mL
Distilled water	80.0	mL

Fixation: Tissues are fixed for up to 24 hours depending on the thickness of specimens

Subsequent: Tissues are transferred into 90% ethanol with several changes of the ethanol solution, followed by absolute ethanol (for the elimination of mercury precipitates see "*Elimination of mercury salts from tissue sections*" or as stated below *Zenker fixative*)

### **Sublimate-formalin fixative (Helly)**

Formalin stock solution (40%)	5.0	mL
Sublimate (HgCl <sub>2</sub> )	5.0	g
Potassium bichromate	2.5	g
Sodium sulfate (cryst.)	1.0	g
Distilled water	100.0	mL

Fixation: Fixative is prepared immediately prior to use by adding formaldehyde to the advanced prepared solution. Tissue blocks (2-4 mm thick) are fixed for up to 6 hours; longer fixation (1-2 days) is usually not harmful

Subsequent: Tissues are transferred into 70% ethanol, followed by 90% ethanol and by absolute ethanol (for the elimination of mercury precipitates see “*Elimination of mercury salts from tissue sections*” or as stated below *Zenker fixative*)

### Sublimate-formalin fixative (Stieve)

Saturated sublimate (aqueous)	76.0	mL
Formalin stock solution (40%)	20.0	mL
Glacial acetic acid	4.0	mL

Fixation: Fixative penetrates tissues rapidly, thus, large specimens can be well fixed; fixation time about 24 hours

Subsequent: Tissues blocks are transferred into 90% ethanol followed by absolute ethanol for dehydration (for the elimination of mercury precipitates see “*Elimination of mercury salts from tissue sections*” or as stated below *Zenker fixative*)

### Sublimate-bichromate-acetic acid fixative (Zenker)

Note: This fixative is a rapid nuclear fixative. The sodium sulfate is frequently omitted. When glacial acetic acid is replaced by formalin, the fixative is called Zenker-formol fixative

Sublimate (HgCl <sub>2</sub> )	5.0	g
Potassium bichromate	2.5	g
Sodium sulfate (cris.)	1.0	g
Distilled water	100.0	mL
Glacial acetic acid	5.0	mL

Preparation of iodine-iodide solution for the removal of mercury salts (sublimate) from fixed tissue blocks.

(a) Iodine/potassium iodide stock solution (mod. Lugol's Iodine):

2.0 g iodine crystals *plus*  
3.0 g potassium iodide are dissolved in  
100.0 mL 90% ethanol

(b) Solution to remove mercury pigment:

several drops of Iodine/potassium iodide stock are added to 70% ethanol until the ethanol solution becomes sufficiently ruby-brown colored (like cognac)

Fixation: Fixative is prepared immediately prior to use by adding glacial acetic acid to the Zenker stock solution. Tissues blocks (2-4 mm thick) are fixed for up to 24 hours

Subsequent: Tissues are washed in water, followed by 70% ethanol, 90% ethanol and, finally, by absolute ethanol

Note: It is advised to eliminate mercury precipitates immediately after fixation by washing tissue specimens in iodine-iodide solution (Lugol's Iodine), i.e. immerse the fixed tissue blocks into the above prepared alcoholic iodine/iodide solution (De-Zenkerization); iodine will remove excessive sublimate by the formation of mercury iodide; ethanol will become discolored. When ethanol remains ruby-brown colored, then sublimate is no longer present and dehydration is continued

De-Zenkerization of tissue blocks is usually not sufficient, and tissue sections need further treatments for elimination of precipitates (see chapter "*Elimination of mercury salts from tissue sections*")

### Zinc fixative (formalin free, sublimate free)

Calcium acetate	0.5	g
Zinc acetate	5.0	g
Zinc chloride	5.0	g
0.1 M Tris-HCl buffer pH 7.4		G

Solution: Mix to dissolve the salts; the final pH will be 6.5-7.0, do not adjust the pH as this will cause the zinc to come out of the solution. Zinc fixative is stored at room temperature

Fixation: Specimens are fixed for 24-48 hours. Dehydration is done in ascending series of ethanol beginning with 50-70% ethanol

### Other fixative solutions

Many other fixatives and fixation schedules can be used for histological purposes. For a detailed description of tissue fixation with special application to electron microscopy see AM GLAUERT (1975) and G MILLONIG (1976).

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