Formalin

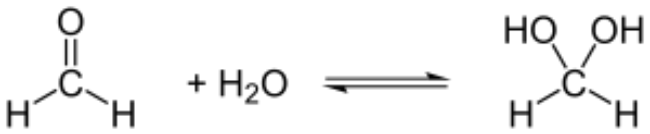
Fixation with formalin is a standard method for conserving tissues for histological and immunohistochemical analysis because it yields superior morphological and microanatomical details. However, it is important to remember that this method may irreversibly modify epitopes recognized by antibodies. This can lead to considerable differences in the reactivity of formalin fixed as compared to frozen tissue sections. Many antibodies react differently and show a loss of reactivity between the two fixation methods, or an altered staining pattern. A few facts may help to understand how to deal with formalin, tissue fixation and immunohistochemistry on formalin-fixed, paraffin embedded tissue sections.

Formaldehyde and Formalin

Formaldehyde is a pungent smelling gas that readily dissolves in water up to a concentration of 37% - 40%. The aqueous solution of formaldehyde is called formalin. Formaldehyde is chemically reactive, and is toxic. Avoid breathing fumes and protect skin and eyes.

Formation of Polymers

Dissolved formaldehyde reacts with water to form methylene glycol, with the equilibrium shifted to the methylene glycol side:



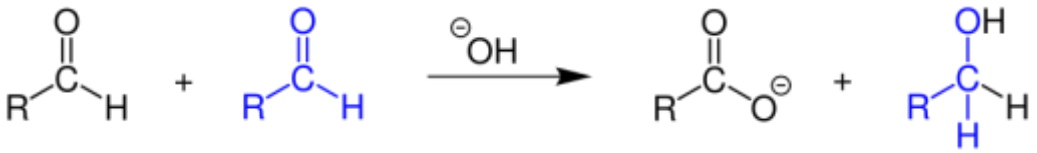
Formaldehyde *Methylene Glycol*

Methylene glycol tends to polymerize into long chains and thereby form an insoluble white precipitate. This precipitate which can often be seen as a white layer on the bottom is called paraformaldehyde. Note that polymerization reduces the number of reactive formaldehyde molecules in solution and thus the power to fix the tissue to be treated.

> Sunlight increases polymerization. Therefore, formalin solutions should be kept in a brown bottle in the dark. The addition of 10%-15% methanol helps to prevent polymerization and formation of precipitate.

Cannizzaro Reaction and Buffered Formalin

Light and heat enable the Cannizzaro reaction, a disproportionation whereby two molecules of formaldehyde form one molecule of formic acid and one of methanol:



Cannizzaro reaction (R = H with formaldehyde)

The formic acid generated in this way decreases the pH and may negatively affect the inegrity of an epitope of interest. Therefore, formalin solutions are generally buffered to achieve optimal tissue fixation. A standard buffered formalin solution can be made up as follows:

Buffered Formalin Solution:

Na_2HPO_4	6.5g (46mM final concentration)
$NaH_2PO_4 \times H_2O$	4.0g (29mM final concentration)
Distilled water	900ml
37%-40% formalin	100ml

Check pH to be 7.4.

> Buffering this solution and keeping it cool and dark increases its lifetime.

Formalin concentrations of 4% versus 10%

The fixation of tissues for immunohistochemical purposes is usually done with a 1 in 10 dilution of aqueous 37% - 40% formaldehyde. This yields a solution containing 3.7% - 4% formaldehyde.
Some protocols consider saturated formalin (37-40%) as „100%“. A 1 in 10 dilution of this solution can be considered “10%”, but actually corresponds to a formaldehyde concentration of 3.7% - 4%.

Mode of Action of Formalin

Formalin enables the cross-linking of proteins with little denaturing. Formaldehyde easily reacts with many chemical groups, with amino groups of particular importance in the context of tissue fixation. Free amino groups, such as the ones found with lysine, are targeted preferentially, leading to altered epitope characteristics.
The duration of the fixation process varies with the nature of the tissue being treated, and with its size. Any length of time from hours to weeks may be ideal and has to be determined empirically.

Improving the reactivity of formalin-fixed tissues

With a little luck, a particular antibody will react comparably with frozen and formalin fixed tissue sections. Sometimes formalin fixed sections may simply need a higher antibody concentration to yield satisfactory results. However, formalin fixed tissue sections often require a particular treatment in order to reverse the effect of formalin fixation, a so-called epitope retrieval process. And many, if not most, epitopes are

irreversibly lost through formalin fixation and require antibodies particularly developed for this application.

Antigen / epitope retrieval techniques

The formalin-induced chemical modification of the epitope recognized by the antibody may be reversed to a variable degree by an antigen (or epitope) retrieval treatment. For proteolytic antigen retrieval, a variety of enzyme solutions such as Proteinase K, pronase, Trypsin, or pepsin may yield optimal results. For heat-induced epitope retrieval (HIER), a microwave oven or pressure cooker is used, where the tissue sections are boiled in citrate or other appropriate buffer. Two standard methods routinely used at BMA are antigen retrieval by heat treatment at low pH, and antigen retrieval by enzymatic digestion with Proteinase K. Keep in mind that different epitope retrieval procedures may yield different reaction patterns with some antibodies.

I. Heat-induced antigen retrieval using microwave oven and citrate buffer

Place slides in a heat resistant holder and put it into a cuvette with 0.01mM citrate buffer, pH 6.0. Boil slides for 2x 7 minutes at 700 Watt in a microwave oven. Refill evaporated buffer after 1x 7 minutes boiling. Cool cuvette to room temperature for 20 minutes (see www.bma.ch →protocols for a detailed protocol).

10mM Citrate buffer:
Dissolve 1.05g Citric acid monohydrate (MW 210.14) in 500ml distilled water, adjust pH to 6.0 with 10M NaOH.

II. Proteolytic antigen retrieval using Proteinase K

Coat the entire section with Proteinase K diluted according to the manufacturer's instructions and incubate for approximately 7 minutes in a humid chamber at room temperature. Incubation time may vary from 3 to 12 minutes depending on the antibody and on the Proteinase supplier (see www.bma.ch →protocols for a detailed protocol).

Proteinase K (BMA Biomedicals, 20mg/ml. Product number T-3401):
Product T-3401 from BMA requires a 1 in 50 dilution for optimal reactivity. Dilution buffer is 20mM Tris-HCl, pH 8.0.

20mM Tris-HCl, pH 8.0:
Dissolve 121mg Tris (MW 121) in 50ml distilled water, adjust pH to 8.0 with 1M HCl.

Combination of heat-induced and proteolytic antigen retrieval

In certain cases a combination of heat-induced and proteolytic antigen retrieval may be beneficial.

A number of factors affect antibody binding in immunohistochemistry. Optimal conditions should be evaluated and optimized for each individual antibody. Every antibody produced by BMA has been validated for its use in immunohistochemistry and data sheets of BMA products indicate if an antibody is suitable for use with formalin-fixed tissues, and which one out of three standard procedures (citrate / proteinase K / no treatment) yields satisfactory results.