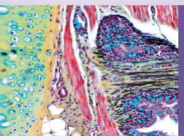

5

Dehydration and Clearing

Chapter Outline

DEHYDRATION

Processing—water is removed (dehydration) from the tissue and replaced by melted paraffin wax. The wax infiltrates the tissue and provides the necessary support when cutting the tissue into thin slices which will eventually be examined under a microscope.

Removal of water is referred to as "dehydration". Tissue processing is done to remove water from the biological tissues, replacing such water with a medium that solidifies, setting very hard and so allowing extremely thin sections to be sliced. This is important because biological tissue must be supported in an extremely hard solid matrix to enable sufficiently thin sections to be cut. Some typical values are:

5 µm thick for **light microscopy;** 5 µm (i.e. 5 micrometers) = 0.005 mm = 0.000005 meter 80–100 nm thick for **electron microscopy;** 80–100 nm (i.e. 80–100 nanometers) = 0.00008 mm to 0.0001 mm = 0.00000008 to 0.0000001 meter.

After a tissue has been suitably fixed, it has to undergo the next step termed *dehydration*, which literally means 'removal of water'. However, the tissues generally contain inside them, two forms of water: *Free* and *bound*. The term dehydration, in its strict sense, is used for the removal of free or extractable water only. The process of dehydration is necessary for two reasons. Firstly, because water is not miscible with paraffin wax in which the tissue will be subsequently embedded for obtaining a paraffin 'block'. Secondly, the water content in the tissue will not be suitable, when its sections will be finally mounted over a slide with the commonly used mountant (Canada balsam) for making permanent preparations. Therefore, all the free water from a fixed tissue needs to be necessarily replaced by a dehydrating fluid during the process of dehydration.

Procedure of Dehydration

A piece of tissue which has been properly washed after fixation is generally transferred to a solution of weak strength of the dehydrating agent (commonly used agent is 30% alcohol). It is then placed successively in solutions of alcohol of gradually increasing strengths (50%, 70%, 90%), ending with two

changes through absolute alcohol. The procedure replaces all the free water by alcohol.

Dehydration process can be started (after fixation) also directly with 90–95 per cent alcohol; but the diffusion-currents that are set up in the passage from water to alcohol may be minimised if the fixed tissue is treated with successive increasing strengths of alcohol.

In dehydrating extremely delicate objects injury to the tissue can be prevented by following special precautions. Such tissues may be placed with some of its liquid in a tube plugged at one end and closed at the other by a diaphragm of some suitable membrane. The tube is then immersed in a vessel containing the desired strength of alcohol. This is allowed to remain like this until, by diffusion through the diaphragm, the two liquids have become of equal density. However, to state that the last alcohol bath should consist of absolutely pure absolute alcohol is incorrect. A strength of 95 per cent (sometimes 90%) alcohol is sufficient in most of the cases. Usually the small amount of water that remains in the tissue after treatment with graded alcohol strengths is satisfactorily removed in the bath of clearing agent to be used after dehydration.

How to Make Different Strengths of Alcohol

In most of the laboratories only two strengths of alcohol are available ready for use. It therefore, often becomes necessary to know as to how various dilutions of alcohol may be prepared. The following schedule, the so-called *dilution method* is recommended.

By this method a particular percentage of alcohol is made by diluting a higher percentage solution of alcohol with the distilled water. In order to obtain a desired percentage from the percentage of alcohol available, the difference in strengths is the amount of water to be added. For example, if 50 per cent alcohol is to be prepared from 95 per cent alcohol; deduct 50 from 95, thus 45 ml will be the quantity of water to be added to the taken 50 ml of 95 per cent alcohol.

For one's own convenience, a dilution table may be prepared for a ready reference (Table 5.1).

Another method for making dilutions of alcohol is depicted in Table 5.2.

Time Required for Dehydration

This depends upon the thickness of the tissue sample. If the tissue is not more than 5 mm in thickness, dehydration may be satisfactorily achieved within 2 to 3 hours. The changes from one bath of alcohol to that of another should be made at an hourly intervals. It is also wise to keep in mind that too long keeping in higher dilutions of alcohol (above 80%) makes the tissue brittle and difficult to cut subsequently. Also, too long keeping in lower dilutions of alcohol (below 70%) should be avoided because this macerates the tissue. Generally a time of one to one and a half hour is sufficient for keeping a sample in the absolute alcohol. The tissue samples should not be left in absolute alcohol for longer time.

Placing the tissue in too much quantity of dehydrating agent causes considerable wastage. Routinely, the volume of dehydrating fluid should be approximately about 10 times the volume of the given tissue.

Substitute for Alcohol in Dehydration

The alcohol is far less satisfactory as a histological preservative, but it acts fairly well as a dehydrating agent. If tissues are placed in alcohol for a prolonged period (weeks and months), the minute structure of the tissues is considerably altered. Tissues get shrunken and too hard. They turn brittle. Their capacity for adequate staining becomes seriously diminished.

Acetone, as a substitute for alcohol is quite frequently used for dehydration purpose due to its very rapid action and relatively less cost. When haste is required, tissues can be well dehydrated with four changes of acetone. Fresh acetone is essential only for the last change.

Table 5.1 Volumes of diluent to be added for making desired strengths of alcohol from alcohol of higher strength

	Percentage strength of original liquid																		
	100	**96**	**95**	**90**	**85**	**80**	**75**	**70**	**60**	**50**	**40**	**30**	**20**	**15**	**10**	**8**	**5**	**4**	**3**
95	5	1																	
90	10	6	**5**																
85	15	11	10	5															
80	20	16	15	10	5														
75	25	21	20	15	10	5													
70	30	26	**25**	**20**	15	10	5												
60	40	36	35	30	25	20	15	10											
50	50	46	**45**	**40**	35	30	25	**20**	10										
40	60	56	55	50	45	40	35	30	20	10									
30	70	66	**65**	**60**	55	50	45	**40**	30	**20**	10								
20	80	76	75	70	65	60	55	50	40	30	20	10							
15	85	81	80	75	70	65	60	55	45	35	25	15	5						
10	90	86	85	80	75	70	65	60	50	40	30	20	10	5					
8	92	88	87	82	77	72	67	62	52	42	32	22	12	7	2				
5	95	91	90	85	80	75	70	65	55	45	35	25	15	10	5	3			
4	96	92	91	86	81	76	71	66	56	46	36	26	16	11	6	4	1		
3	97	93	92	87	82	77	72	67	57	47	37	27	17	12	7	5	2	1	
1	99	95	94	89	84	79	74	69	59	49	39	29	19	14	9	7	4	3	2

Volumes of diluent fluids to be added

Note: a. **Heavy type** figures indicate strengths of alcohol most commonly required for use.
b. Underline figures indicate most common strengths of formaldehyde. Formalin is 40% formaldehyde.

Table 5.2 Dilution table for alcohol

To obtain alcohol at	80%	70%	60%	50%	40%
Add to 100 ml of 95% alcohol the following ml of water	21 ml	39 ml	63 ml	96 ml	144 ml

CLEARING OR DEALCOHOLISATION

After a tissue is dehydrated (usually with alcohol) the next step in the process of paraffin block-making is called as '*clearing*'. The object of clearing is to remove alcohol before impregnation of tissue is started. The process of clearing is, therefore, sometimes called *dealcoholisation*.

The clearing agents (dealcoholisation agents or antemedia) are liquids whose function is to make tissue transparent. These agents are capable of raising the refractive index of any specimen soaked in them. This makes the tissue transparent (clear); and the agents are sometimes referred to as *clearer*. Majority of clearing agents are essential oils and themselves have high refractive index. Besides being able to dealcoholise, the clearing agents also facilitate the penetration, into tissues, of paraffin wax in which the tissue is to be embedded; and of Canada balsam in

which final mounting is to be done. However, it is important to note that all clearing agents cannot be used as true clearers. For example, although glycerine is a clearing agent, it is neither suitable for making paraffin blocks, nor it can be satisfactorily used if sections are to be finally mounted in balsam. This is so because glycerine is not miscible with either of them. On the other hand, chloroform is an admirable clearing agent (precursor of paraffin and Canada balsam), but can hardly be utilized as a clearer because the desired transparency of tissue is not achieved.

Selection of a Clearing Agent

The selection of a clearing agent depends generally on the type of preparation of a histological section. Isotonic saline is quite useful for temporary preparations of animal tissues. For permanent preparations, however, xylene is the most frequently used clearing agent probably since it serves the purpose both in embedding and mounting. Toluene is relatively less harmful than xylene because it does not cause any hardening of the tissue; but at the same time clears less rapidly than xylene or chloroform. Chloroform also does not harden like xylene or toluene, but it has a very poor penetration in the tissue, hence not preferred as a clearing agent. For the best work on animal tissues the clearing agent of choice is cedar-wood oil. Table 5.3 depicts merits and demerits of some commonly used clearing agents.

Replacement of Water by Clearing Agents

Good quality clearing agents, specially oils, are capable of removing small quantities of free water that is left in the tissue even after the end of dehydration process. The cedar-wood oil will remove water from tissues saturated

Table 5.3 Merits and demerits of some commonly used clearing agents

Name of the clearing agent	Merits	Demerits
Xylene	• Can be used as clearing agent both in embedding and mounting • Rapid in action • Does not dissolve celloidin • Staining with aniline is not affected	• Prolonged use over hardens and shrinks the tissue • Inadequate dehydration causes the formation of a whitish emulsion
Cedar-wood oil	• Can be used to clear both paraffin and celloidin sections • Penetration is well • No shrinkage caused • Immersion in cedar-wood oil improves section cutting • Tissues may be left in it indefinitely	• Clearing is slower than xylene • Sections tend to retain traces of oil till mounting, hence a quick dip in xylene becomes essential • Treatment with xylene necessary prior to paraffin impregnation • Quite expensive
Benzene and toluene	• Less hardening and shrinkage than xylene	• Emulsification occurs even with slightest traces of water
Chloroform	• Hardening almost nil as compared with xylene and toluene	• Penetration rate is very slow • Adequate transparency is not achievable
Dioxan	• Clearing possible even if tissues are not put into alcohol solutions (it is also capable of dehydrating tissues) • Prolonged immersion does not damage tissues	• Extremely toxic to nasal mucosa and conjunctiva

with 95% alcohol. Oils of bergamot and aniline oil are capable of such removal from the tissues saturated with 90 and 70% alcohols respectively.

With certain precautions, tissues may be made dehydrated satisfactorily even without the use of alcohol at all. For routine work, the usual commercial aniline will suffice. Even if it has turned brown through oxidation aniline (otherwise colorless) can still be utilized. However, for better clearing, perfectly anhydrous oil is required.

In routine histological procedures the term clearing may be used in two forms.

Clearing in Embedding

In this form of clearing, the tissue is transferred to a clearing agent after being adequately dehydrated. Since the tissue is to be embedded in paraffin wax, the clearing agent used here should be readily miscible with alcohol and also must be a solvent of paraffin. The examples of such clearing agents are: Xylene, dioxan, chloroform, and cedar-wood oil.

Clearing in Mounting

Although this form of clearing would be required after the sections have been stained and again dehydrated, one should be familiar with clearing in mounting. It also means dealcoholisation, not of the tissue but of the section. Since the stained sections are ready to be mounted, the clearing agents, employed for clearing in mounting must not only be capable of dealcoholisation but they must also be solvents of mounting media (usually Canada balsam). The examples of this category of clearing agents are: Xylene, toluene, and benzene.

The common practice of taking out the tissue out of the alcohol and then placing it on the surface of a cleaning agent is faulty. The reason for this is that the alcohol escapes from the surface of the tissue sample into the air quicker than the clearing agent can get into it. This causes tissue shrinkage. To prevent or at least to minimize the shrinkage, it is advisable that a small quantity of clearing agent must be placed under the alcohol containing the

Mixtures for clearing		Time periods up to which tissue is to be kept in the mixtures	
		Bulk tissue	Sections
Mixture—I (25: 75)			
Clearing agent	25 ml	30 minutes	2 minutes
Alcohol (absolute)	75 ml		
Mixture—II (50: 50)			
Clearing agent	50 ml	30 minutes	2 minutes
Alcohol (absolute)	50 ml		
Mixture—III (75: 25)			
Clearing agent	75 ml	30 minutes	2 minutes
Alcohol (absolute)	25 ml		
Pure clearing agent		Till transparent	Till transparent

Note: If clear-wood oil is used as a clearing agent, do not shake the oil with the alcohol. Gently add the alcohol to the oil and drop the tissue in it. As the tissue is penetrated with oil, it will sink. The time for clearing bulk tissues varies with different clearing agents, and also with the size of the specimen being processed. Generally a period of about half an hour is sufficient for clearing. However, 24 hours may be considered as the maximum time required for clearing.

tissue in it. The word 'under', used here, needs to be little clarified. The clearing agent may be put 'under' the alcohol by following one of the two methods.

1. Alcohol is taken in a tube; the tissue to be cleared is put in it; and then a sufficient quantity of clearing agent is introduced, at the bottom of alcohol, with the help of a pipette.
2. First the clearing agent is poured in a tube; then alcohol is gently added on the top of the agent. After this, the specimen to be cleared is carefully transferred into the supernatant alcohol. Gradually the specimen sinks and at the same time the two fluids (alcohol and clearing agent) mix together slowly. After the specimen gets sunk to the bottom of the tube, alcohol is gently drawn off with a pipette.

Technique and Stages of Clearing

When any tissue gets completely cleared, it appears transparent. In the process the tissue is passed through mixtures of alcohol and clearing agent as per the following schedules.

SUMMARY

Dehydration

Definition: Removal of water and fixative from tissue, and replaced them with dehydrating fluid.

- For this purpose alcohols of various strengths are used.
- As alcohols are hydrophilic in nature, it drags water from tissues by diffusion.
- So the increasing concentrations starting from 50%, 70%, 90% and two replacements of absolute alcohols are used.
- For delicate tissues like embryos, animal tissue start concentration with 30% dilution.

Clearing (Dealcoholization)

Clearing is the next step after dehydration. It removes alcohol from the specimen before the impregnation starts. The agents used for clearing are called *antemedia*; and these raise the refractive index of the tissue to make it transparent (clear)—hence the name of the process. Commonly used clearing agent is **xylene**. For merits and demerits of some other commonly used clearing agents refer to Table 5.3.

Usage of the Term Clearing

The term clearing is used in *two* forms:

i. ***Clearing in Embedding***—after dehydration, the specimen is transferred to a clearing agent that should be clearly miscible both with alcohol and solvent of paraffin wax.

ii. ***Clearing in Mounting***—means dealcoholization of a stained 'section'—not of specimen