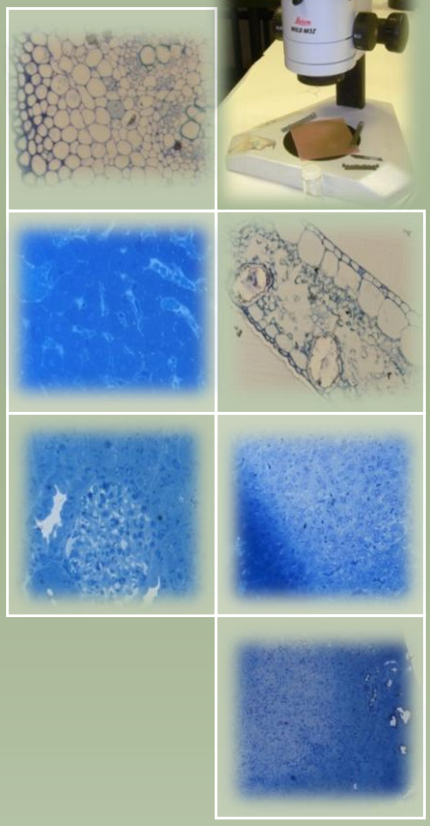
2009 - 2010



Electron Microscopy Unit

Kuwait University, Faculty of Science

Biological Sample Preparation for TEM Observation

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Under the Supervision of

Dr. Ali Bumajdad

Director of the EMU

Before You Start:

Kindly read our rules and regulations, as they are in favor of your benefit

- Every user has to fill a complete online application form from our website.

- Upon approval of the application by the Director of the unit, the user can submit his/her samples (not before that)

- All samples should be registered in the unit TEM registration book and given a serial number. (first come, first served)

- No samples are to be submitted to the unit for fixation on Thursdays, as no staff is available on week ends to change the fixative.

- Fixatives and buffer solutions are supplied to users upon prior request.

- It is advisable that the chemical reagents used for sample preparation to be prepared by the unit technical staff.

- The user should be aware that a reasonable amount of samples are grouped to be processed in the Tissue Processor in order to save effort, time and chemicals. So it is not a daily or an individual process.

- The unit will hold no responsibility of unsuccessful sample preparation carried out by the user and loss or mislabeling of specimens.

- Do not hesitate to seek the help of the technical staff in the unit in case if any problem arises.

Safety

Kindly read all the safety precautions before starting the preparation steps

1- Almost all chemical reagents used in the preparation for EM observation are toxic, so they should be handled carefully in a fume hood and the user should wear latex gloves, lab coat and a mask.

2- Fixatives are poisonous and volatile, so stock and waste fixative bottles should not be open for a long time.

3- Osmium tetroxide vapor is very hazardous to eyes and the respiratory system, so put on a mask and gas-tight goggles.

4- During preparation and use of resins, it is advisable not to breathe the vapor and to avoid spills and contact with skin as they are toxic and some are carcinogenic.

Note: in case of any spill, please wear gloves while cleaning the area with ethanol. In the absence of gloves, clean hands with soap or detergent, because ethanol facilitates absorption by skin.

5- Waste resin may be disposed in a closed container in a fume hood and when full can be cured to polymerize in an oven at 70˚ C.

6- All other waste toxic chemical reagents are to be disposed following the local safety routine of the Faculty of Science. Please contact the safety officer for further information.

(Do not dispose toxic solutions in the sink)

7- Always make sure that all tools used in preparation are clean and when necessary tools should be sterilized in an autoclave for delicate microbiology work.

8- Kindly clean the used glassware, and remove any remnants of chemical solutions or dirt from benches.

Tools

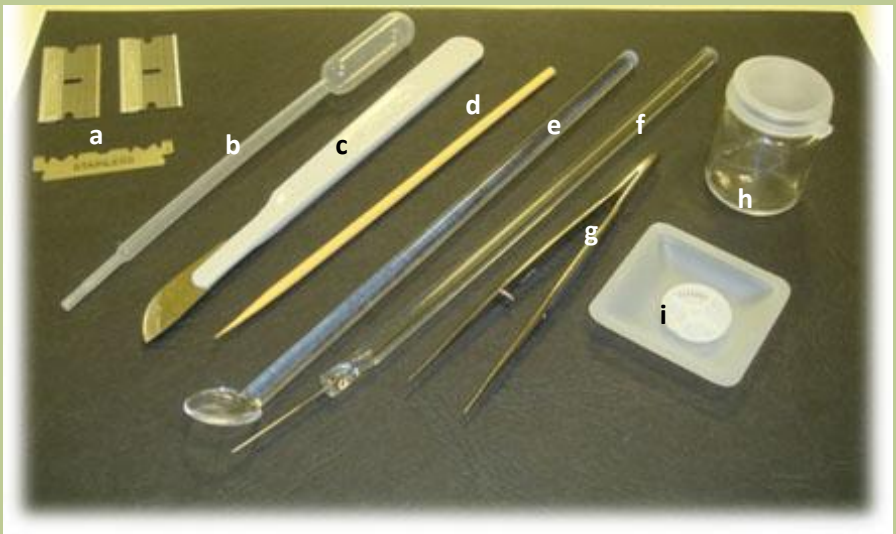


Figure 1: Tools used in sample preparation: from left to right (a) blades, (b) polyethylene transfer pipette, (c) scalpel, (d) bamboo stick, (e) glass rod, (f) dissecting pin, (g) forceps, (h) glass vial with cap and (i) weighing boat with tissue processor basket.

- Blades, single edge, double edge or scalpel
- Dissecting pin.
- Pasteur pipettes and rubber bulbs.
- Polyethylene transfer pipettes.
- Tissue identification labels.
- Specimen vials with caps.
- Amber glass bottles.
- Plastic bottles.
- Petri dishes.
- Dental wax.
- Bamboo sticks.
- Glass rods.
- Forceps.
- Syringes.
- BEEM capsules.
- Flat embedding molds.
- Weighing boats.
- Beakers.
- Measuring cylinders.
- Latex gloves.
- Goggles or protective glasses.
- Masks.

Equipment

- Balance, top loading (Mettler Toledo).
- Fume Hood.
- Oven (Fisher Scientific).
- pH meter (WTM).
- Stereo Microscope (Intralux 4000 1).
- Tissue Processor (Leica- Reichert LYNX)
- Tissue Rotator (Ted Pella, Inc).

Figure 2: Equipment used in biological preparation lab: (a) balance, (b) oven, (C) stereo microscope, (d) tissue processor, and (e) tissue rotator.

Chemicals

- Glutaraldehyde.
- Phosphate buffer.
- Osmium tetroxide.
- Ethanol.
- Propylene oxide.
- Epon resin.
- Distilled water.

Figure 3: Chemical solutions (a) Osmium tetroxide, (b) phosphate buffer and (c) glutaraldehyde fixative.

Figure 4: Epon component bottles and a top loading balance.

Preparation of Chemical Solutions

Fixatives:

Glutaraldehyde: 3%

1. *Combine 12ml (25%) glutaraldehyde and 88ml phosphate buffer.*
2. *Check pH , it should be 7.2-7.4*
3. *Store in the refrigerator.*
4. *Discard the fixative after one month.*

Buffers:

Millonig's Phosphate Buffer:

1. *Dissolve 1.8 g of sodium orthophosphate dibasic ($NaH_2PO_4.2H_2O$), 23.25g of disodium orthophosphate monobasic ($Na_2HPO_47H_2O$) and 5.0g of sodium chloride (NaCl) in distilled water with a final volume of liter.*
2. *Check pH, it should be 7.4*

Cacodylate Buffer 0.1mol/L

1. *Dilute 21.4g sodium cacodylate to 900mL with distilled water.*
2. *Adjust pH to 7.3 with concentrated hydrochloric acid.*
3. *Dilute to 1000mL with distilled water.*
4. *Keep in the freezer in plastic bottles.*
5. *Let buffer thaw and shake well before use.*

Osmium Tetroxide 2% in Buffer

(Preparation must be carried out in a fume hood)

1. *Wash the vial containing osmium tetroxide (OsO_4) crystals with detergent to clean it and remove the label.*
2. *Place the vial in a clean amber glass bottle containing 50 ml of buffer solution (phosphate buffer).*
3. *Place the lid on the bottle and shake sharply to break the vial.*
4. *Allow vial contents to dissolve completely.*
5. *Keep the bottle in refrigerator.*
6. *Osmium tetroxide can be filtered before use to get rid of the broken glass.*

Epoxy Resins:

Epon (812):

Epon 812 epoxy monomer	*27.1g*
MNA hardener	*15.3g*
DDSA hardener	*7.6 g*
DMP accelerator	*0.5-0.75ml*

Note: hardness is controlled by changing amount of hardeners

Araldite:

Araldite M	*10.1m*
Araldite hardener	*10.0m*
Araldite accelerator	*l0.3m*
Dibutyl Phthalate	*l0.6ml*

Spurr:

ERL4206 epoxy monomer	*10.0g*
DER736 flexibilizer	*6.0g*
NSA hardener	*26.0g*
S-1 accelerator	*0.4g*

Note: hardness is controlled by amount of DER736 (4-8)

1. *Weigh components accurately in a disposable plastic bottle or container on top-loading scale.*
2. *Add the components in the same order as listed above except the accelerator.*
3. *Mix well with glass rod (avoid mixing with wooden sticks).*
4. *Use pipette to add the few milliliters of the last component and mix well.*
5. *Allow to stand for 10 minutes to get rid of bubbles.*

Note: It is preferred to prepare resins the same day to be used and the remaining resin should be kept in a plastic container in refrigerator at 4ºC for several days or in a freezer for weeks or months. At the time of reuse, allow to thaw at room temperature to avoid any water contamination.

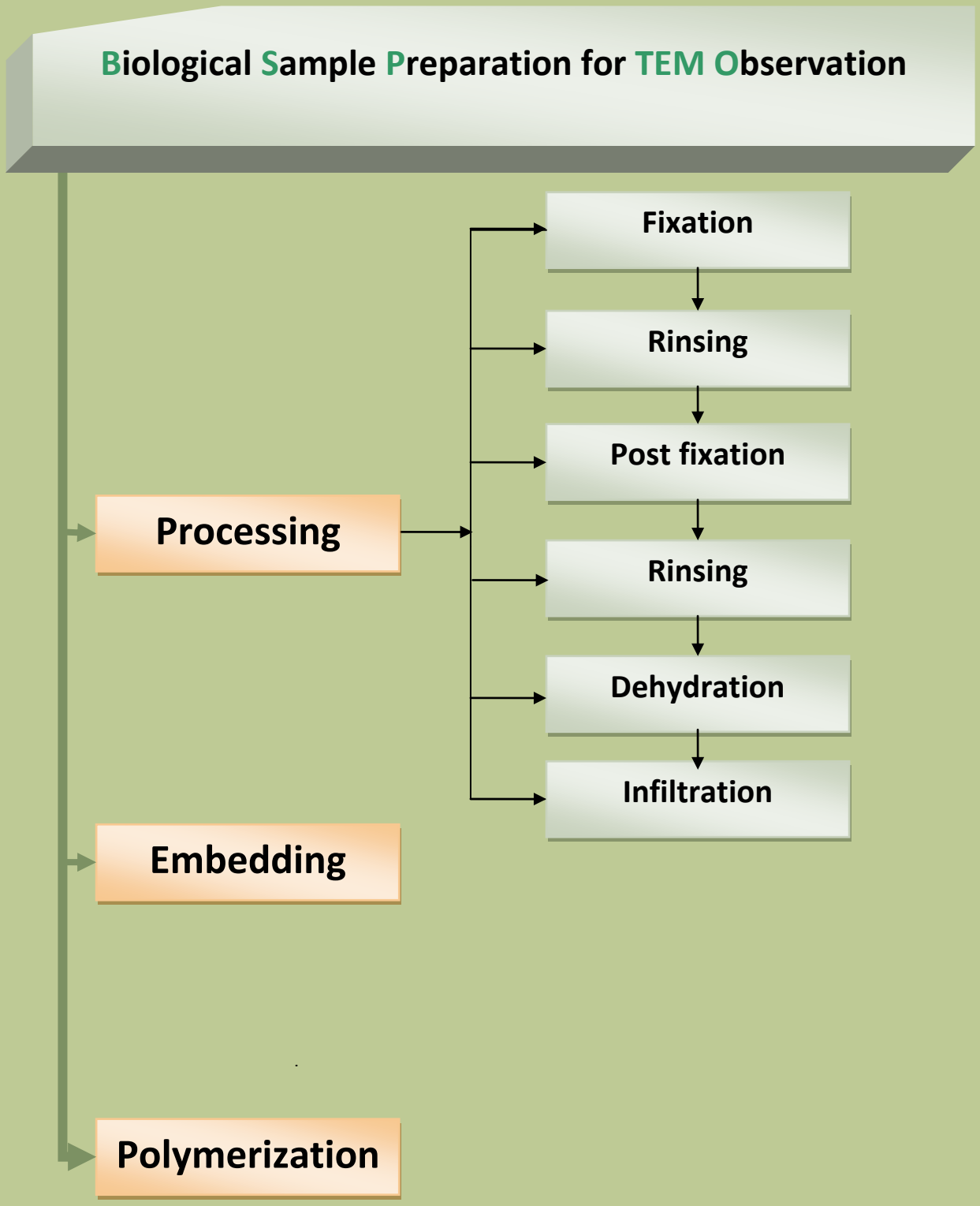


Figure 5: Flow chart of sample preparation steps

Method

Processing

The following steps are carried out manually:

Note: use different Pasteur pipettes for different solutions

Chemical Fixation:

This step is very critical to preserve the cellular structure with the minimum alteration of morphology, volume and spatial relationship.

For Animal Tissues:

1. Gently pick specimen with forceps and dip it in a pool of glutaraldehyde fixative on a clean sheet of dental wax.
2. Immerse the specimen completely with fixative with Pasteur pipette.
3. Cut the specimen to thin slices (1mm) or small blocks (0.5-1mm^3) with a double edge blade, broken into two halves or a scalpel (this could be done on the stage of a stereo microscope to identify the area of interest of the specimen).

Note: the cutting should be in a slicing motion to prevent damage to the tissue

4. Immerse the specimen pieces quickly in the labeled glass vials containing fixative (*fixative should be at least ten times the size of the specimen*).
5. Allow the specimen to be fixed at room temperature for 1-2 hrs using tissue rotator, then keep overnight in the refrigerator.

For Plant Tissues:

1. Place fresh sample in a Petri dish with filter paper wetted with fixative or in a pool of fixative on a clean sheet of dental wax.
2. Cut the sample to 1cm slices with single blade, then to $1mm^3$ pieces with double edge blade.
3. Transfer the specimen pieces with forceps to the labeled glass vial containing fixative.
4. If a plant tissue floats to the surface of the fixative, it should be vacuum fixed to remove the gases in order to allow good penetration of fixative solvents and resins as follows:
   place the glass vial in a vacuumed oven at 25-30 psi until gas bubbles are released and the tissue pieces sink to the bottom of the vial. (discard all floating specimens).
5. Keep the specimen in fixative 1-2 hrs at room temperature then place in refrigerator overnight.

For Microbiological Cells:

*a- **Cells Grown on Agar Plate:***

1. Cut with a single blade or scalpel small pieces from an area with excessive amount of cells.
2. Immerse gently in glass vial containing fixative.
3. Keep in fixative 1-2 hrs at room temperature then transfer to refrigerator overnight.

*b- **Cells in Suspension:***

1. Centrifuge sample to allow accumulating at the bottom of the tube.
2. Pipette off the solution from the tube and with a clean pipette add fixative.
3. Keep in fixative 1-2 hrs at room temperature then transfer to refrigerator overnight.

In some cases, delicate cell samples on agar or in suspension are processed manually on a rotator as they do not withstand the agitation of auto-processing. Solutions could be added to cells on agar in Petri dish.

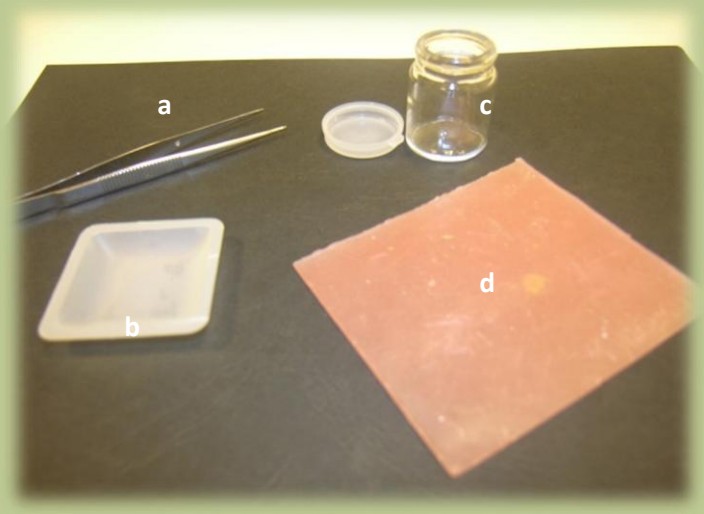


Figure 6: Tools used for fixation: (a) forceps, (b) weighing boat, (c) glass vial with cap and (d) dental wax.

Rinsing:

Sample tissue should be washed with a solvent for fixative namely; a buffer to maintain pH and tonicity

1. Take the vials from the refrigerator.
2. For the 0.5mm^3 specimen blocks, Pipette off the fixative not letting the specimen to be completely dry. For 1mm slices, place the specimen into fixative on dental wax and cut them to smaller blocks (0.5mm^3) and then transfer tissue to vials containing phosphate buffer.
3. Wash the specimen pieces in three changes of buffer, for ten minutes each time, using Pasteur pipette. Place the vials on a rotator.

Note: make sure to check any tissue adhering to the pipettes after use in different specimen vials to avoid mix up between specimens.

Post Fixation:

Secondary fixation with osmium tetroxide, to increase contrast and stability of fine structure

1. Pipette off the buffer from the vials.
2. Replace with osmium tetroxide and keep for 2 hrs on a rotator.

Rinsing:

Wash the specimens in three changes of buffer to get rid of all remains of osmium tetroxide.

Dehydration:

The epoxy resins used in infiltration and embedding are not miscible with water, so the water content in tissue should be replaced with an organic solvent, ethanol is used in increasing concentrations.

Infiltration:

Epoxy resin is used to penetrate the cells and fill the spaces in between to give hard plastic material that will tolerate the pressure of cutting.

Resin is not miscible with water so a transitional solvent is used like propylene oxide

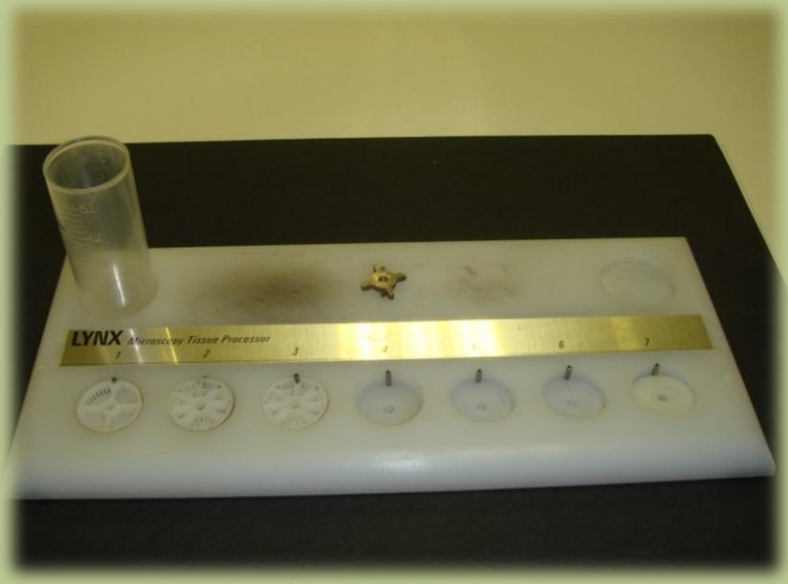
Note: in case of hard plant tissue, Spurr resin is used

***Dehydration and Infiltration** are carried out in the Leica Tissue Processor as follows:*

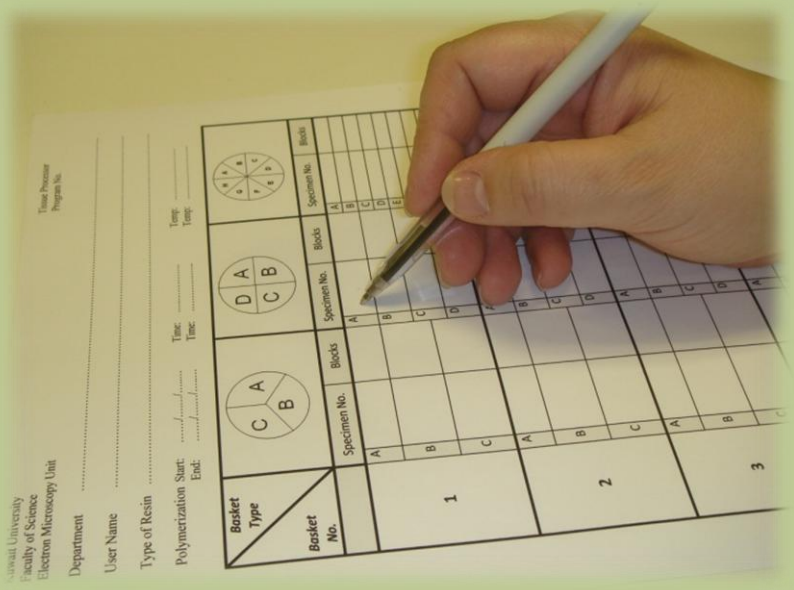
Loading the Specimens:

1. Place the required number and type of baskets in the loading jig cavities.

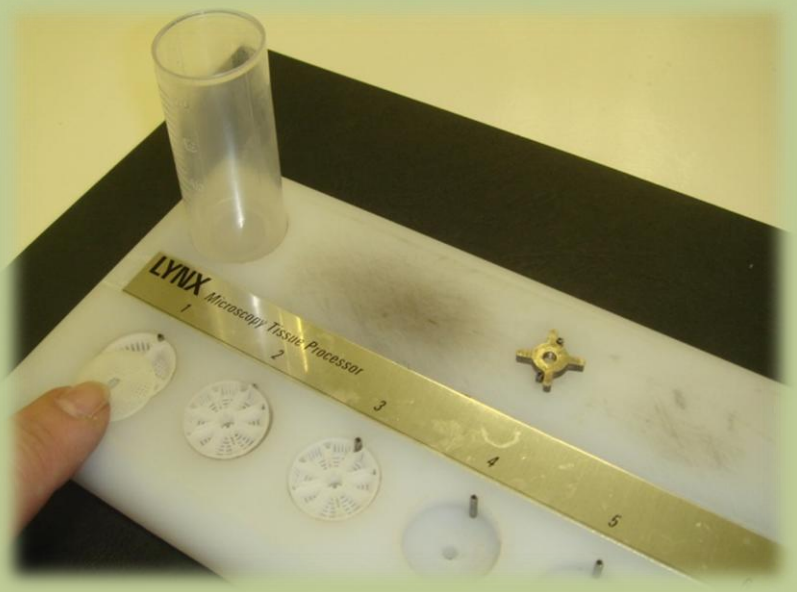
2. Place the threaded foot between the pins.



3. Transfer the specimens to the baskets and record the locations.



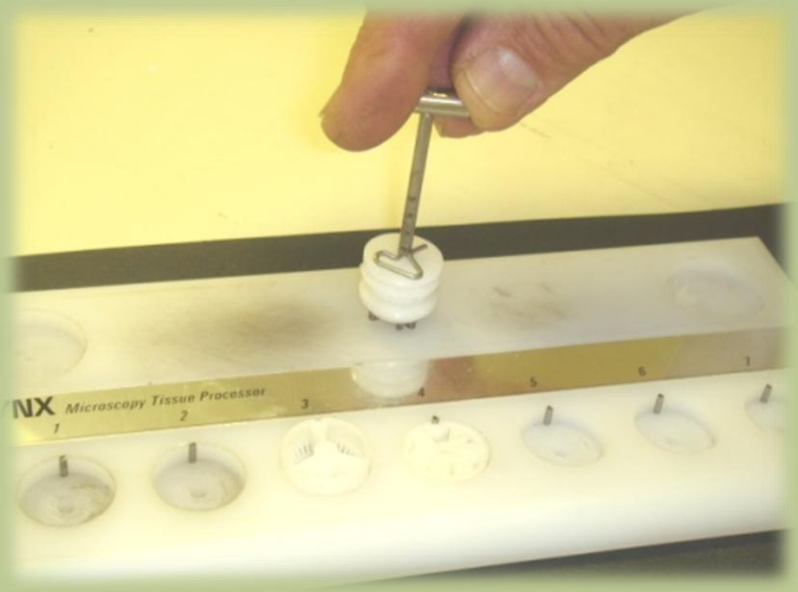
4. If Fresh specimen is used, flood the basket completely with buffer.
5. Cover the baskets with the lids.



6. Insert the clip into the required position on the stem.

7. Pick up the baskets with lids on the stem.

8. Secure the stack with the threaded foot.



9. Keep the baskets and stem assembly in a vial filled with buffer.

Filling the Vials:

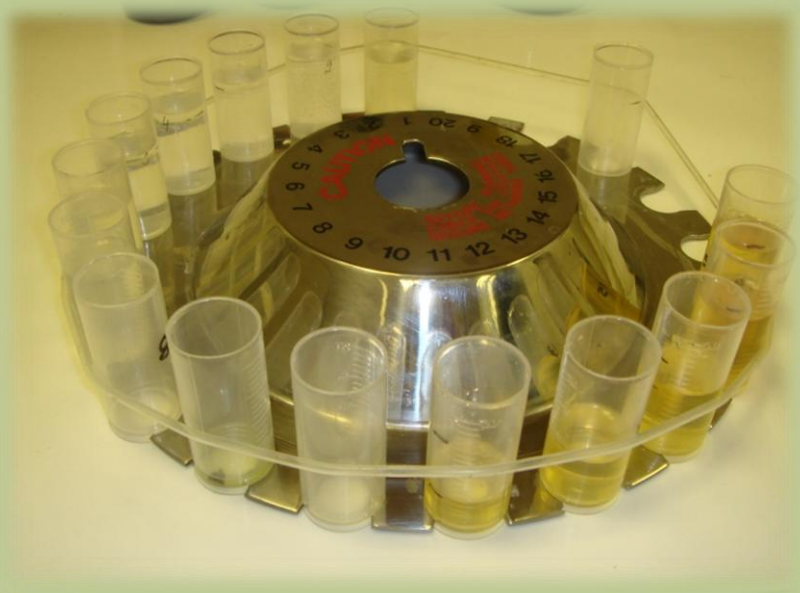
1. Removing the turntable:

- Unscrew the central retaining cap and lift the turntable clear of the locating pin.

- Rotate the turntable and lift it clear.

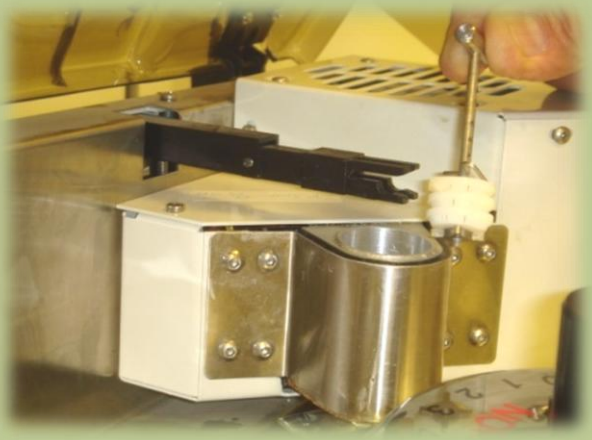
2. Fill the vials with the required volume of the reagents to a maximum of 15ml.
3. Fit the vials to the turntable positions required for the selected program (no.4) and press firmly until the vial meets the edge of the turntable.

4. Fit the vial retaining band to the base of the vial.



5. Replace the turntable and secure into position on the locating pin with the central screwed cap.

6. Transfer the baskets and stem assembly to the tissue processor agitation arm without delay and lower the arm into position manually.



7. Place the vial lid around the stem above the baskets, with the raised ridge of the vial lid fitting into the hole at the top of the heater/cooler unit.

8. Secure the vial lid, close the tissue processor lid and start the program.

Running the Program:

The program followed in this preparation method is No.4

Set the key switch on the right side of the tissue processor to:

PROGRAM PROTECT.

1. Review the program steps.
2. RUN the program.
3. At the end of the program, press STANDBY.

Unloading Specimens:

1. Raise the tissue processor lid and remove the vial lid.
2. Release the stem assembly from the agitation arm, lower it into the vial, and press LOWER.
3. Unscrew the central retaining cap, rotate the turntable and lift clear.
4. Dismantle the basket and stem assembly in the reverse order of the loading procedure.
5. Place each basket containing the specimens on a weighing boat labeled with the same basket number.

Embedding

1. Prepare the molds, usually BEEM capsules, but for specimens with special orientation, flat molds are used. Examine molds to be certain there is no debris or lint present.
2. Write the identification information required by the unit on labels with pencil or type this information and then make a photocopy (ink dissolves in resin).
3. Cut the labels into small squares that fit in the bottom of the flat molds, and roll the labels for capsules and insert them against the inside wall with the writing facing outside.
4. Pour little amount of resin in each mold using polyethylene transfer pipettes or syringes.
5. Uncover the basket and carefully pick the specimen with forceps and blot it at the rim of the weighing boat and place it either in the center of a BEEM capsule or at the tip of a flat mold.
6. Allow specimens to sink in the bottom of BEEM capsules and fix the orientation of specimens in flat molds under a stereo microscope with a straight pin.
7. Fill the molds completely with resin avoiding any spill.
8. Get rid of air bubbles in each mold in order to provide a smooth cutting procedure of the block later.

Note: Resin can be placed in an oven 2-3 minutes at 70˚C to reduce viscosity which makes it easier to handle.

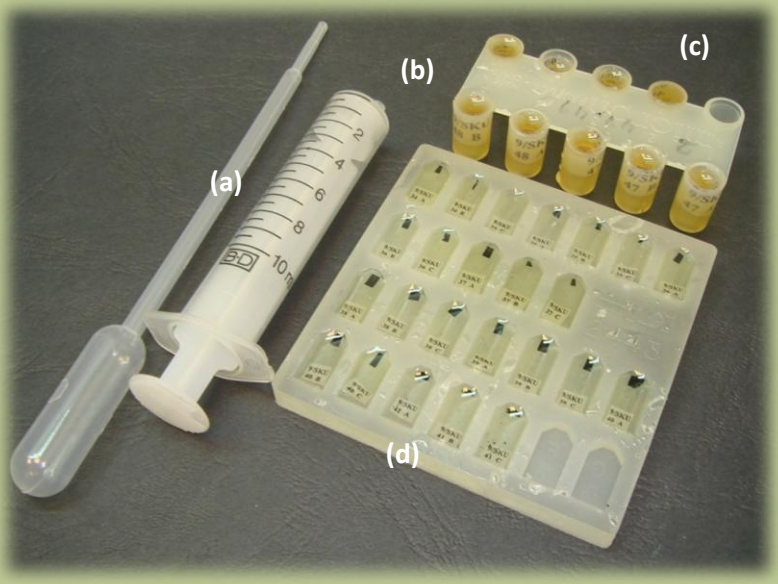


Figure 7: Tools used for embedding: (a) Polyethylene transfer pipette, (b) syringe, (c) BEEM capsules and (d) flat molds.

Polymerization

1. Check the orientation of the specimens.
2. Place the molds containing specimens in an oven at 70˚C for three days when using Epon and Araldite resins and 9 hours when using Spurr resin.
3. Stop curing the blocks by programming the oven with the following code **(.)** (Day) (1) (Time) (05) C

Importance of Fixation

FIXATION is the most critical step in this long and interdependent method, so if it is not carried out successfully then continuation of the following steps is useless. Poorly fixed samples will not be cut smoothly and may disintegrate in the knife water bath. Sections might stain unevenly for light microscopy observations and thinner sections may disrupt under the high voltage beam of TEM. Examples of well and poorly fixed animal and plant tissue sections are shown in Figures 8, 9 and 10.

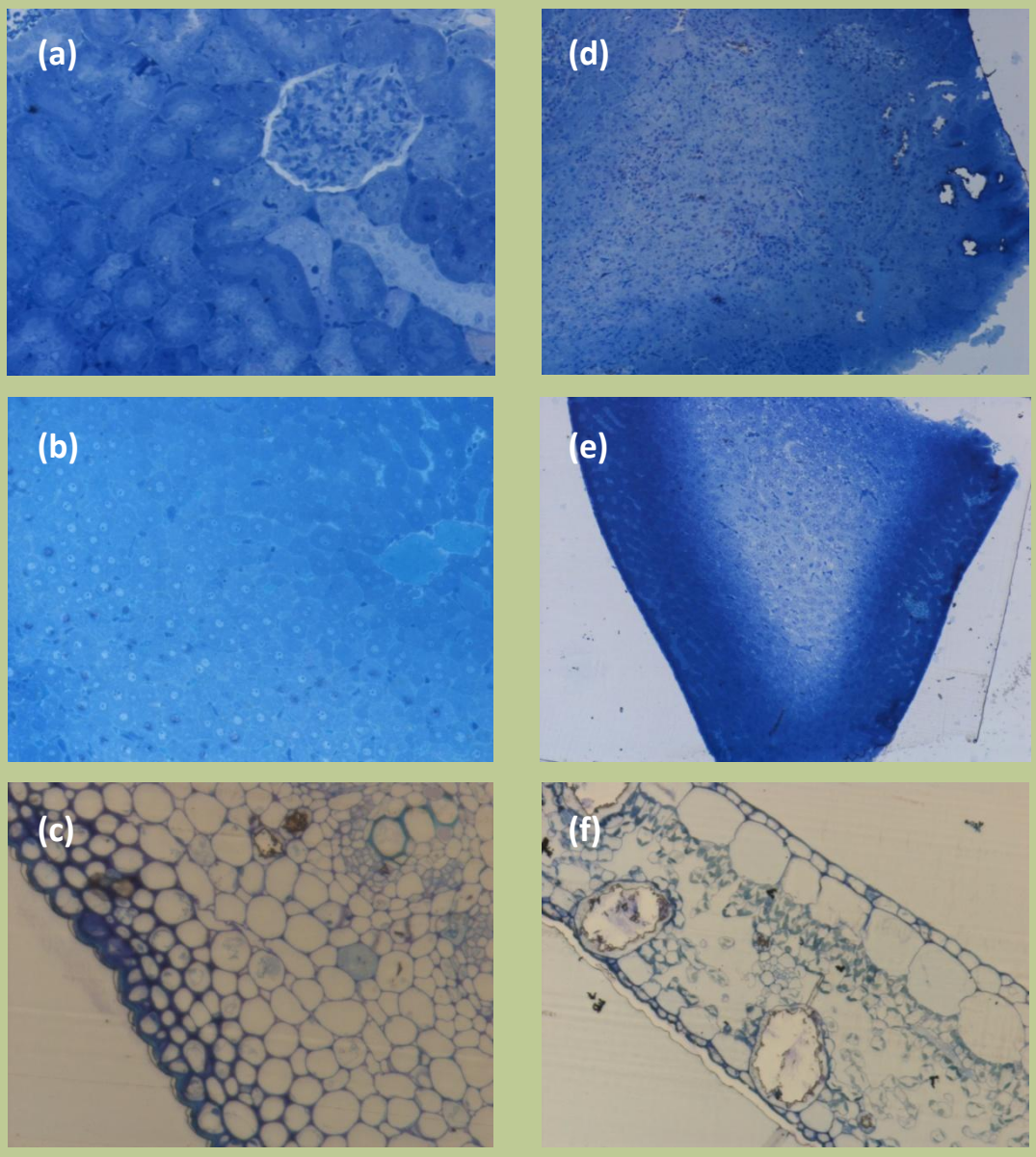


Figure 8: Light microscope photomicrographs showing well fixed: (a) kidney, (b) liver and (c) plant Leaf and poorly fixed: (d) kidney, (e) liver and (f) plant Leaf. Sample and photo courtesy by: Ahlam AlKadi, 2009.

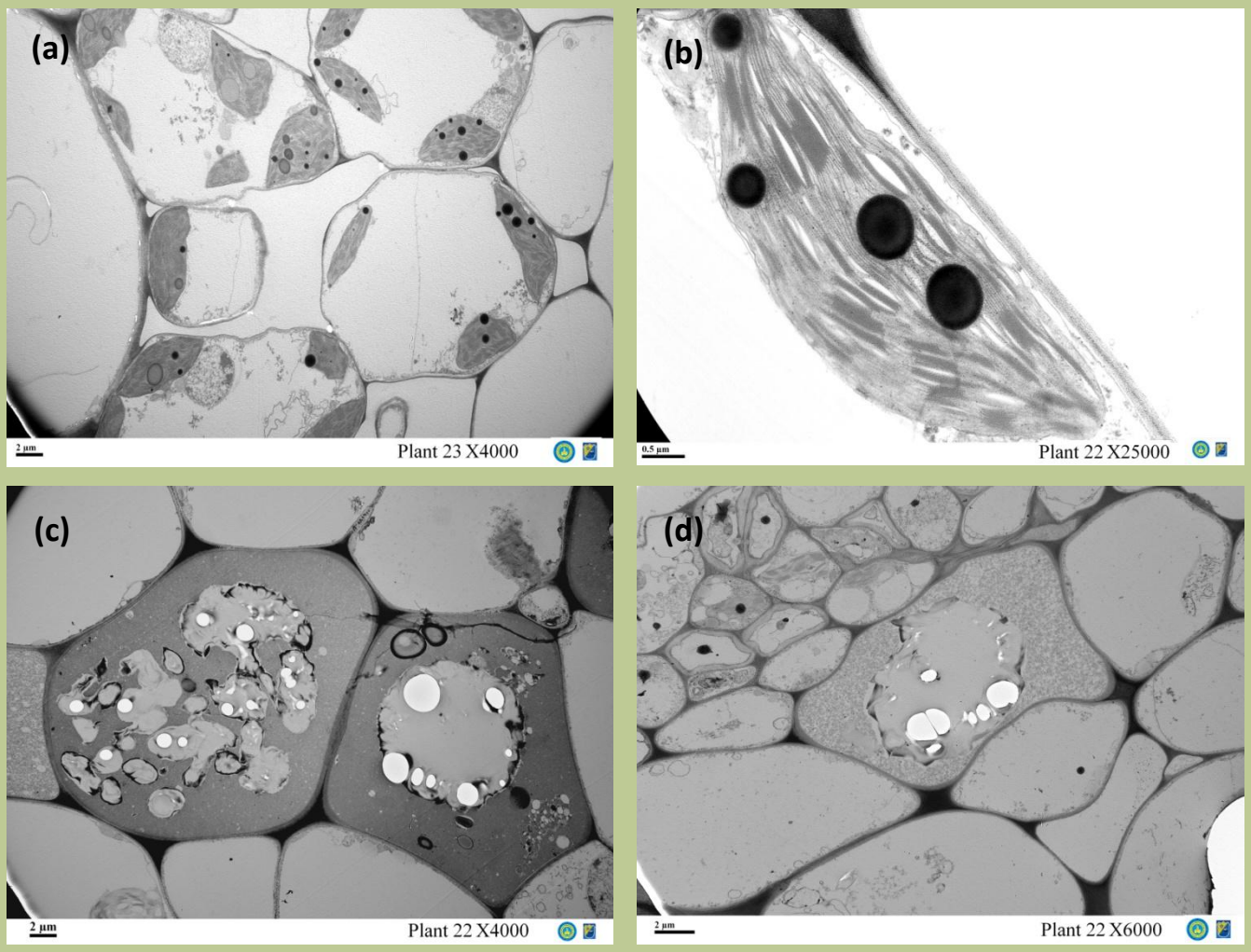


Figure 9: TEM photomicrographs showing well fixed: (a) plant leaf, (b) chloroplast and poorly fixed: (c, d) plant leaves. Sample and photo courtesy by: Ahlam AlKadi, 2009.

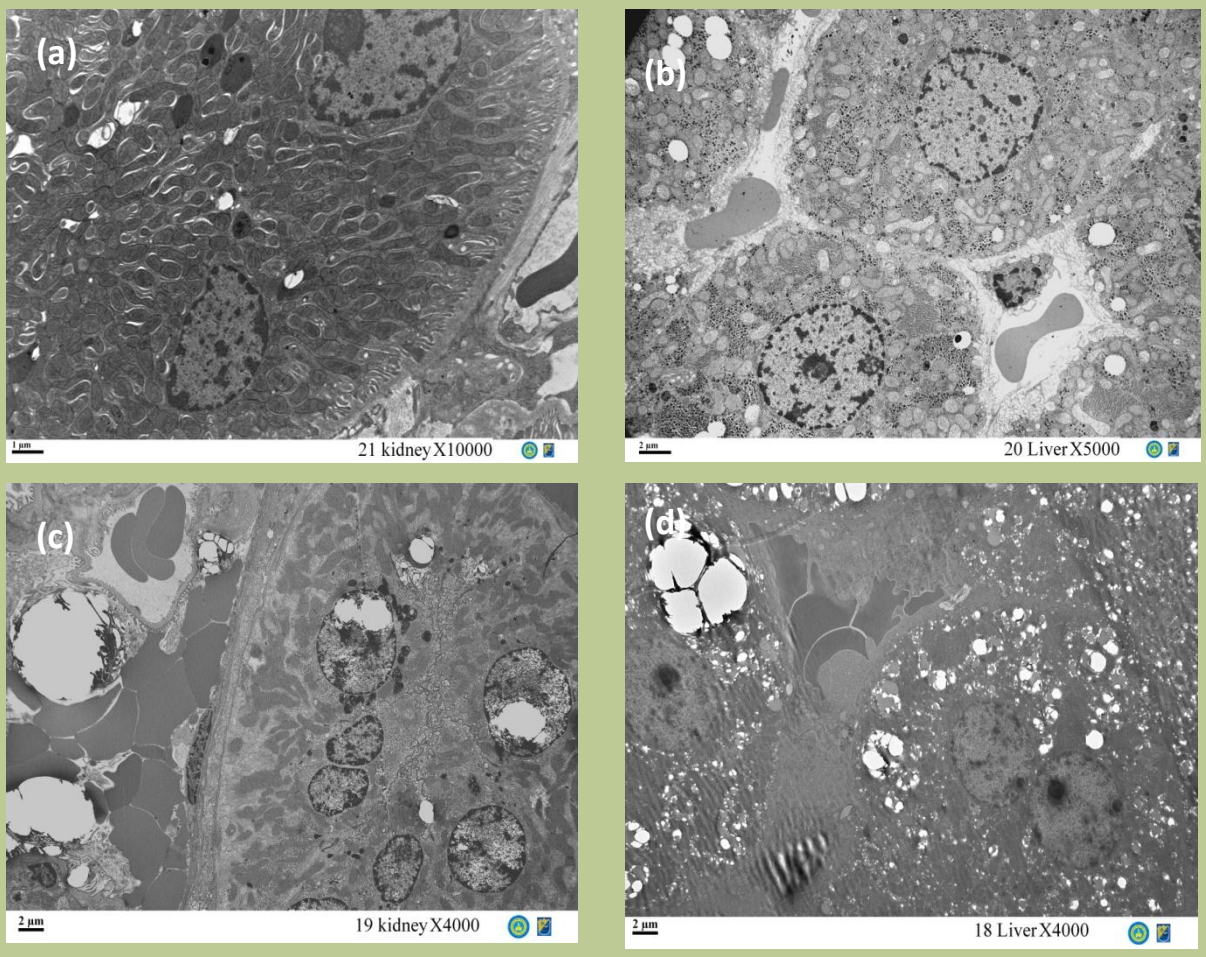


Figure 10: TEM photomicrographs showing well fixed (a) kidney, (b) liver and poorly fixed (c) kidney and (d) liver. Sample and photo courtesy: by Ahlam AlKadi, 2009.

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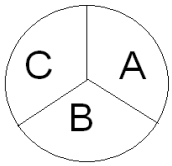
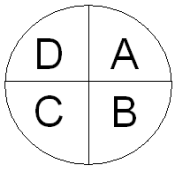
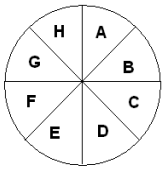
Appendix A (Tissue Processor Record Sheet)

Kuwait University
Faculty of Science
Electron Microscopy Unit

Tissue Processor
Program No.

Department ……
User Name ……
Type of Resin ……
Polymerization Start: ……/……/……. Time: …..…….. Temp: …….
End: ……/……/……. Time: ……..….. Temp: ……..

Basket Type / Basket No.						
	Specimen No.	Blocks	Specimen No.	Blocks	Specimen No.	Blocks
1	A		A		A	
					B	
	B		B		C	
					D	
			C		E	
	C				F	
			D		G	
					H	
2	A		A		A	
					B	
	B		B		C	
					D	
			C		E	
	C				F	
			D		G	
					H	
3	A		A		A	
					B	
	B		B		C	
					D	
			C		E	
	C				F	
			D		G	
					H	
4	A		A		A	
					B	
	B		B		C	
					D	
			C		E	
	C				F	
			D		G	
					H	

Appendix B (Tissue Processor Program 4)

Step No.	Processing Steps	Recommended time	Vial No.	Temp °C	Agitation
Washing					
1	**Wash** (buffer) 1	10 min.	1	20	**applied**
2	**Wash** (buffer) 2	10 min.	2	"	"
Dehydration					
3	Alcohol 50%	10 min.	3	"	"
4	Alcohol 60%	10 min.	4	"	"
5	Alcohol 70%	10 min.	5	"	"
6	Alcohol 80%	10 min.	6	"	"
7	Alcohol 90%	10 min.	7	"	"
8	Alcohol 100%	10 min.	8	"	"
9	Propylene Oxide 1	10 min.	9	"	"
10	Propylene Oxide 2	10 min.	10	"	"
Infiltration					
11	Propylene oxide+Resin(2:1)	60 min.	11	"	"
12	Propylene oxide+Resin(1:1)	60 min.	12	"	"
13	Propylene oxide+Resin(1:2)	60 min.	13	"	"
14	Pure Resin Mixture 1	6 hrs.	14	"	"
15	Pure Resin Mixture 2	8 hrs.	15	"	"
	Total Time	**18 hrs.40 min.**			

Appendix (C): poster (1)

ROUTINE BIOLOGICAL SAMPLE PREPARATION PROCEDURE FOR TEM OBSERVATION AND IMAGE ACQUISITION

To view a biological sample in the TEM, a strong, well processed, accurately cut, well stained ultrathin section of the sample is prepared following this procedure in the EMU.

Specimen Preparation

- **1- Processing**
  - Manual: using a tissue rotator
  - Auto: using a tissue processor

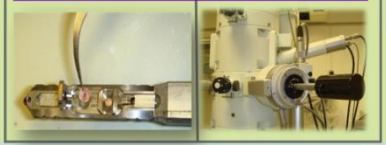
  Includes: a-Fixation, b-Rinsing, c- Post Fixation, d- Rinsing, e- Dehydration, f- Infiltration
- **2- Embedding**
  - BEEM capsules for nonoriented samples.
  - Flat molds for oriented samples.
- **3- Polymerization**
  - Curing the resin containing the sample in an oven set at temperature and time depending on type of resin.

Ultramicrotomy

- **1- Knife Preparation**
  - Using the knife maker to prepare glass knives and the Reickert multiplate to fix plastic truffs with dental wax.
- **2- Trimming**
  - Using single edge blades to remove excess resin over sample and trim the sample face to trapezium shape.
- **3- Cutting**
  - Using glass or diamond knife to cut sections by Ultramicrotome.
- **4- Staining**
  - Using chemical dyes to increase contrast during observation.

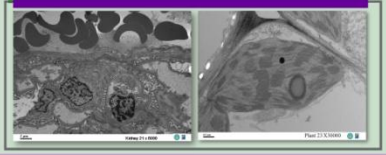
Semithin sections 1µm thick collected on glass slides and stained with Toluidine Blue using hot plate.

Ultrathin sections 70-100 nm thick collected on Cu or Al grids and stained with uranyl acetate and lead citrate using ultrostainer.



Observation

- **Light Microscopy**
  - Semithin sections are observed under light microscope or Coolscope
- **Transmission Electron Microscopy**
  - Ultrathin sections on grids are loaded in TEM sample holder and observed under the microscope.



Photography

- **Analog Camera**
  - Pictures are captured on Kodak plate films by TEM built in camera
- **Digital Camera**
  - Pictures are captured by attached Gatan CCD camera

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Poster (2)


Kuwait University, Faculty of Science, Electron Microscopy Unit
BIOLOGICAL SAMPLE PREPARATION FOR TEM OBSERVATION
Ahlam Alkadi and Sami Hassounah
Introduction
Over the last few years, advances in sample preparation techniques for electron microscopy work have been enormous both in terms of (1) availability of a wide range of histological protocols that deal with complex biological tissues and (2) the supportive instrumentation used to accomplish delicate steps in preparation such as specimen coating. As commonly employed, temporary protocols used for routine laboratory work result in poor preservation of both surface structure (topography features) and ultrastructure (cellular details) of biological tissues. Add to this the rapid deterioration of tissue contents resulting from prolonged storage (> 1 hour) which restrict researcher work. It is becoming increasingly important, therefore, to employ physical (i.e. application of heat or cold treatments) and chemical fixation techniques as to preserve tissues and cease its biological activity for long term investigations. The most common physical fixation techniques used in electron microscopy work involve heat (Microwave) and cold (Cryofixation) treatments, while chemical fixation techniques involve the use of varying penetrative fixatives such as aldehyde, Osmium tetroxide, formaldehydes, permanganates and glutaraldehydes. One crucial step in sample preparation protocols is the addition of buffer, nonelectrolyte and electrolytes agents to a selected fixative as to overcome problems resulting from tonicity and pH. The latter tend to stabilize surface structure and cellular components of specimens hence better details are revealed by SEM and TEM, respectively. One of the major core facilities offered by the Electron Microscopy Unit (EMU) at Kuwait University is the SAMPLE PREPARATION LABORATORY which is equipped with necessary instrumentation used for fixation, dehydration, embedding, sectioning and staining of thin sections and surface coating of a variety of biological specimens. The objective of the current practical training session is twofold: to provide EMU users with hand skills on biological sample processing techniques commonly used in electron microscopy work and to highlight significant role of tissue preservation to proceed with advanced electron microscopy work such as immunolabelling and microanalysis techniques.
Equipments Recommended for Biological Samples Preparation in the EMU.
In order to study a biological sample in the Transmission Electron Microscope, a special EM protocol has to be carried out. It starts with the first and most important step: Sample Preparation and ends with the Preparation of a Section of the sample. This section should hold the following specifications: (a) to preserve the same cellular ultrastructure of the original sample, (b) to be of an extremely thin thickness (70-100nm) to allow the electron beam to pass through and strong enough to withstand the high vacuum in the microscope and (c) it should be stained to yield good contrast to give a high quality image.
Tools Required for Biological Samples Preparation.
SAMPLE PREPARATION METHOD
SAMPLE PROCESSING
EMBEDDING
To embed the sample in molds filled with resin
POLYMERIZATION
To cure the resin in an oven at 70°C in order to provide solid blocks containing the sample to be sectioned
Flat molds
BEEM Capsules
FIXATION
RINSING
POST FIXATION
RINSING
DEHYDRATION
INFILTRATION
FIXATION is the most critical step in this long and interdependent method so if it is not carried out successfully then continuation of the following steps is useless. Poorly fixed samples will not be cut smoothly, may disintegrate and in the water bath, stain unevenly and disrupt under the beam in the TEM. Examples of well and poorly fixed animal and plant tissue sections are shown in Figure 1.
Figure 2: Chemicals, Tools and Various Steps of Sample Preparation in the Tissue Processor.
Figure 1: Effect of fixative on plant and animal tissues: (a) well fixed plant leaf, (b) a fractured poorly fixed plant leaf, (c) well fixed liver tissue, (d) unevenly stained poorly fixed liver tissue, (e) well fixed kidney tissue, (f) a fractured and unevenly stained poorly fixed kidney tissue. Sections were prepared, stained with Toluidine Blue and photographed using the Goolscope microscope by Ahlam Alkadi, 2008.
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