Tissue Processing
Dehydration to Plastic

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Abstract

Tissue preparation in the electron microscopy lab to use TEM to look at the vitreo-retinal interface in eyes.

§1 Introduction

This document is intended to be used to process tissue from formalin to embedded plastic to be used on the transmission electron microscope (TEM) to identify the orientation of collagen fibers.

§1.1 Sorting

Begin first by sorting the tissue in two piles of tissue that was peeled and tissue that was adjacent to the peeled region. Then write down the identification ID # on the paper to keep the proper vial straight during the tissue process.

§1.1.1 Identification ID #

Sheep #, L/R, E/P, P/A

For example, *UL-15A-B Left Equator Peel* can be reduced to *UL15LEP*

§2 Dehydration

First place samples in glass vials. Use forceps if it is required to remove excess waste from the container. Properly label the samples from before section 1.1.1 and place the label on the vial. Before adhering the label to the vial, write down the number of specimens in the vial to ensure that the specimens don't get lost during the process. Use tape to ensure that the label will not be removed from the vial during the process.

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§2.1 Buffer Rinse

Remove the fixative from the existing vial using the micropipette. Be sure not to suck out the tissue. Then fill the vial with buffer - 0.1M Sodium Cacodylate buffer.

§2.1.1 Agitation

Put the sample vials in the rotating agitator for 5 minutes.

§2.2 Buffer Rinse

Remove the buffer from section 2.1 and replace with new buffer - 0.1M Sodium Cacodylate buffer.

§2.2.1 Agitation

Put the sample vials in the rotating agitator again for 5 minutes.

§2.3 Osmium dilution

During the previous agitation step in section 2.2.1 dilute the osmium tetroxide OsO_4 (4% in dH_2O) with 0.2 M Sodium Cacodylate buffer in a 1:1 mixture. Be sure to filter the Osmium tetroxide with a millipore filter to remove any excess particulate that would otherwise result in artifacts inside the tissue.

§2.4 Osmium rinse

Remove the 0.1M Sodium cacadylate buffer from the vials and replace with the diluted Osmium from section 2.3. Use just enough diluted Osmium to cover the tissue.

§2.4.1 Agitation

Put the sample vials back in the rotating agitator again for one hour.

§2.5 DI water rinse

Remove the diluted Osmium tetroxide from the vials and rinse with DI water. The DI water will be filtered [1]. This step is done to remove excess osmium.

§2.5.1 Agitation

Put the sample vials back in the rotating agitator again for 5 minutes.

§2.6 Uranyl Acetate rinse

Remove the DI water from the vials and replace with Saturated 4% Aqueous Uranyl Acetate. The Uranyl Acetate also needs to be filtered using a millipore filternote1 on a 10 ml syringe.

§2.6.1 Agitation

Put the sample vials back in the rotating agitator again for one hour.

[1] The millipore filter is used to remove any excess particulate that would otherwise result in artifacts inside the tissue.

§3 Final acetone dehydration step

The final step of the dehydration process is to replace all of the moisture in the tissue from H_2O to pure acetone. This is done with a series of rinses in various percentages of alcohol with the last set of rinses in acetone. **Note - if there is not enough alcohol mixtures in the hood then you will need to make more. When making the dilutions, use the graduated cylinder that is in the sink and mix the highest concentrations first to ensure that the percentages of alcohol is correct. Start with 95 then 70 then 50 etc. Also be sure that the ethanol containers are covered to prevent evaporation during each step of the dehydration process.

§3.1 50% Ethanol Alcohol

Remove the urinal acetate from the vial in section 2.6 to the appropriate container. Next use the micropipette and fill the vial with 50% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.1.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.2 70% Ethanol Alcohol

Remove the 50% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 70% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.2.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.3 95% Ethanol Alcohol

Remove the 70% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 95% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.3.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.4 95% Ethanol Alcohol

Remove the 95% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 95% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.4.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.5 100% Ethanol Alcohol

Remove the 95% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 100% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.5.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.6 100% Ethanol Alcohol

Remove the 100% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 100% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.6.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.7 100% Ethanol Alcohol

Remove the 100% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 100% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.7.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.8 100% Ethanol Alcohol

Remove the 100% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with 100% Ethanol Alcohol; ensure that the tissue specimen is well covered.

§3.8.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.9 Acetone

Remove the 100% Ethanol Alcohol from the vial. Next use the micropipette and fill the vial with acetone; ensure that the tissue specimen is well covered.

§3.9.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.10 Acetone

Remove the acetone from the vial. Next use the micropipette and fill the vial with acetone; ensure that the tissue specimen is well covered.

§3.10.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.11 Acetone

Remove the acetone from the vial. Next use the micropipette and fill the vial with acetone; ensure that the tissue specimen is well covered.

§3.11.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§3.12 Acetone

Remove the acetone from the vial. Next use the micropipette and fill the vial with acetone; ensure that the tissue specimen is well covered.

§3.12.1 Agitation

Put the sample vials in the rotating agitator again for 10 minutes.

§4 Infiltration

Once the tissue samples have been completely dehydrated and all moisture in the sample has been replaced with acetone, the next step is to infiltrate with plastic. This will allow the tissue to be embedded and then cut using the Ultramicrotomes. This will also take a few steps that still incorporate various mixtures of acetone and plastic.

§4.1 Acetone & Plastic

The first step is to remove the acetone from the vial using a micropipette and replacing it with a 1:1 mixture of acetone and plastic. Again, as mentioned before, the vial does not need to be filled up to the brim, just enough to throughly allow plastic to infiltrate the tissue.

§4.1.1 Agitation

Put the sample vials in the rotating agitator again for one hour.

§4.2 Acetone & Plastic Overnight Option**

If you are to finish the process for the day and return the next, then perform the following option, if not skip to section 4.3. First remove the 1:1 mixture from section 4.1 and replace with a 3:1 mixture of plastic to acetone and let it sit overnight.

§4.3 Acetone & Plastic

If you are to finish the process the same day then skip section 4.2. First remove the 1:1 mixture from section 4.1 and replace with a 3:1 mixture of plastic to acetone.

§4.3.1 Agitation

Put the sample vials in the rotating agitator again for one hour.

§4.4 Pure Plastic

First remove the 3:1 mixture from either section 4.2 or 4.3 and replace with pure plastic.

§4.4.1 Agitation

Put the sample vials in the rotating agitator again for one hour.

§4.4.2 Vacuum

Place all of the vials with the lids removed inside the vacuum chamber. Turn the pump on to remove air from the chamber. This will remove all air from the samples that has been embedded inside the tissue and will allow the infiltration of plastic to fully take affect. Let the samples sit inside the vacuum chamber for one hour.

§4.5 Pure Plastic

Remove the pure plastic from section 4.4 and replace with pure plastic again.

§4.5.1 Agitation

Put the sample vials in the rotating agitator again for one hour.

§4.5.2 Vacuum

Place all of the vials with the lids removed inside the vacuum chamber. Turn the pump on to remove air from the chamber. This will remove all air from the samples that has been embedded inside the tissue and will allow the infiltration of plastic to fully take affect. Let the samples sit inside the vacuum chamber for one hour.

§5 Embedding

The next step is to embed the plasticized tissue into the mold. Before forgoing with this process, a list of all of the specimens will need to be created on Excel to print and cut out. For example if there are five specimens in the same vial, make a list of sample names with the specimen ID (A), specimen ID (B), ... specimen ID (E). Next, grab a razor blade and a wooden stir stick. Simply use the razor blade to shave away wood from the stir stick to make a flat surface. The flat surface will be used to transfer specimens from the vials to the mold. Place the printed out label inside the mold and set the mold inside the oven to let it bake the specimens to cure the plastic.

§6 Cutting

After the plastic has cured, remove the specimen to be cut and use the microtome to shave away thin layers to be used for TEM.

§7 Grid Staining

Once thin sections have been placed on grids from section 6 the grids will need to be stained to increase the contrast for TEM. Two chemicals will be Uranyl Acetate and Lead Citrate.

§7.1 Preparation

Using the square petri-dish and wax from the cupboard cut the wax to fit the inside the petri-dish. Clean the wax with alcohol and DI water to remove any impurities on the wax that would alter the grid samples. This will also prevent the drops from coagulating together on the wax. Simply rinse the wax to clean it off.

§7.2 Chemical Prep

After the wax has been cleaned and cut remove the saturated Uranyl Acetate and Reynold's Lead Citrate from the refrigerator. Grab two small 1 ml syringes from the drawer and fill up each syringe with either UA or Lead Citrate. Then place one small filter on the end of the syringe filled with UA and two filters on the syringe filled with Lead Citrate.

§7.3 UA Stain

Using the 1 ml syringe with a single filter place a droplet of UA for each grid that you need to stain evenly spaced on the wax pad. Use the forceps and remove the grids from the grid holder and place on top of the UA droplet. Be sure to place the grid shiny side down to allow the UA to stain the specimen.

§7.4 Timer - 18 minutes

Set the timer for 18 minutes. During this time fill up enough 30 ml syringes with DI water for rinsing both UA and Lead Citrate. You will need approximately 10 ml per sample per rinse. Place a large filter on the end of the syringe.

§7.5 Staging Area

Grab a small round petri dish and insert two filter papers to absorb the water following the rinse. Use a pen or pencil to mark the paper to help organize the order of specimens to prevent a mix up.

§7.6 First Rinse

After 18 minutes, pick up the grid with forceps and rinse with 10 ml of DI water. Hold the forceps at a 60° angle from the horizontal and drip the water down the curved section of the forceps. After the rinse, place the specimens inside the round petri dish to remove excess DI water. Once all of the specimens have been placed on the filter paper, a few sodium hydroxide crystals will need to be placed inside the square petri dish. The NaOH will help prevent any sort of moisture from interfering with the grid during the staining process. Next, use the other 1 ml syringe with Lead Citrate and place drops on the wax pad following the same procedure mentioned before in section 7.3.

§7.7 Lead Citrate Rinse

Using the forceps, grip the grid and place it on top of the Lead Citrate droplet with the shiny side down which allows the grid to be stained. Set the timer for eight minutes.

§7.8 Second Rinse

After eight minutes have passed, repeat the same step as in 7.6. Once the grids have completely dried, place them back in the grid holder and they are ready for the TEM.

§7.9 Cleanup

Dispose of the petri dish in the unwanted UA container.

§8 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through it.

§8.1 TEM

Head over to the TEM and begin imaging!

#	Step	Instruction	Time	☑	☑	☑	☑	☑	☑	☑	☑
1	Dehydration	0.1 M Sodium Cacodylate buffer	*A** 5 minutes								
2	Dehydration	0.1 M Sodium Cacodylate buffer	*A** 5 minutes								
3	Fix	4% OsO_4 with 0.2 M Sodium Cacodylate buffer (1:1 filtered)	*A** 60 minutes								
4	Rinse	DI water rinse	*A** 5 minutes								
5	Stain	Saturated 4% Aqueous Uranyl Acetate (filtered)	*A** 60 minutes								
6	Dehydration	50% Ethanol	*A** 10 minutes								
7	Dehydration	70% Ethanol	*A** 10 minutes								
8	Dehydration	95% Ethanol	*A** 10 minutes								
9	Dehydration	95% Ethanol	*A** 10 minutes								
10	Dehydration	100% Ethanol	*A** 10 minutes								
11	Dehydration	100% Ethanol	*A** 10 minutes								
12	Dehydration	100% Ethanol	*A** 10 minutes								
13	Dehydration	100% Ethanol	*A** 10 minutes								
14	Dehydration	Acetone	*A** 10 minutes								
15	Dehydration	Acetone	*A** 10 minutes								
16	Dehydration	Acetone	*A** 10 minutes								
17	Dehydration	Acetone	*A** 10 minutes								
18	Infiltration	1:1 Plastic to Acetone	*A** 60 minutes								
19	Infiltration	3:1 Plastic to Acetone	*A** 60 minutes								
20	Infiltration	Pure Plastic	*A** 60 minutes								
21	Vacuum	Vacuum	*V** 60 minutes								
22	Infiltration	Pure Plastic	*A** 60 minutes								
23	Vacuum	Vacuum	*V** 60 minutes								
24	Embedding	Embedding	Limitless								

Table 1: Simplified instructions to check off the steps during the tissue processing by hand. *A** indicates Agitation, and *V** indicates Vacuum.

Station #	Step	Instruction	Time	☑	☑	☑	☑
1	-	-	-	-	-	-	-
2	Dehydration	0.1 M Sodium Cacodylate buffer	*A** 10 minutes				
3	Fix	4% OsO_4 with 0.2 M Sodium Cacodylate buffer (1:1 filtered)	*A** 60 minutes				
4	Rinse	DI water rinse	*A** 10 minutes				
5	Stain	Saturated 4% Aqueous Uranyl Acetate (filtered)	*A** 60 minutes				
6	Dehydration	50% Ethanol	*A** 10 minutes				
7	Dehydration	70% Ethanol	*A** 10 minutes				
8	Dehydration	95% Ethanol	*A** 10 minutes				
9	Dehydration	95% Ethanol	*A** 10 minutes				
10	Dehydration	100% Ethanol	*A** 10 minutes				
11	Dehydration	100% Ethanol	*A** 10 minutes				
12	Dehydration	100% Ethanol	*A** 10 minutes				
13	Dehydration	100% Ethanol	*A** 10 minutes				
14	Dehydration	Acetone	*A** 10 minutes				
15	Dehydration	Acetone	*A** 10 minutes				
16	Dehydration	Acetone	*A** 10 minutes				
17	Dehydration	Acetone	*A** 10 minutes				
18	Infiltration	1:1 Plastic to Acetone	*A** 60 minutes				
19	Infiltration	3:1 Plastic to Acetone	*A** 60 minutes				
20	Infiltration	Pure Plastic	*A** & *V** 60 minutes				
21	Infiltration	Pure Plastic	*A** & *V** 60 minutes				
22	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-

Table 2: Simplified instructions to check and make sure the automatic tissue processor is set up at the correct stations. Each vial should be filled with 20 ml when processing. Be sure to check the program on the automatic tissue processor; it should be marked by program #2. *A** indicates Agitation, and *V** indicates Vacuum.