© 2017 by John N. Anderson, All rights reserved

ModernBio SERIES
BY JOHN N. ANDERSON

IND-26 • INSTRUCTOR GUIDE
Localizing Tubulin by Immunohistochemistry

I. Chemicals and Materials Provided

	Amount
Trachea/Esophagus Slides	8
Clearing Agent (Histoclear II	100ml
Mounting Medium (Histomount)	10ml
Coverslips	1 pack
Slide Staining Dishes	9
Large Transfer Pipets	20
Small Transfer Pipets	30
Glass Rods	8
30 X TBS	25ml
Alpha tubulin antibody	1ml
Second antibody	1ml
Triton X 100	1ml
Peroxidase Substrate	1ml
*BSA Blocking solution	1.4ml
Hematoxylin	1ml
Eosin	1ml

The BSA blocking solution contains 3% BSA (bovine serum albumin), 0.15 M NaCl and 10 mM Tris, pH 8.0 and the antibodies are diluted in BSA Blocking solution.

The tubulin antibody is a mouse monoclonal antibody while the second peroxidase labeled antibody is directed against the tubulin antibody. The second antibody is an Anti-mouse IgG-peroxidase antibody produced in goat. The peroxidase substrate contains tetramethylbenzidine (TMB) and hydrogen peroxide. TMB has been - reported to be a noncarcinogenic substrate of peroxidase - See: V. Holland, Tetrahedron, 30, 3299, (1974)

II Storage - All items should be stored at room temperature except the BSA blocking solution and antibodies, which should be stored frozen.

II. Preparation of Solutions

Please note: High quality distilled water should be used for all solutions and for the water washes described in the student manual. Ethyl alcohol or isopropyl alcohol (95-100%) are needed but not provided for this experiment.

To prepare the 70% alcohol solution, add 90 ml of water to 210 ml of the alcohol.

To prepare the 30% alcohol solution, add 210 ml of water to 90 ml of the alcohol.

To prepare the working solution of TBS (Tris Buffer saline), add the 25 ml of 30 X TBS water to 725 ml of distilled water. The working solution contains 0.15 M NaCl and 10 mM Tris, pH 8.0.

To prepare the working solution of Triton X100, add 0.5 ml o the concentrate to 100 ml of distilled water. The working solutio contains 0.5 % Triton X100.

All other solutions are shipped ready to use.

III. Prelab Preparation

The instructor or one student in the class should arrange the solutions in the sequence indicated below. Beakers or simila containers holding sufficient distilled water to cover a slid submerged from the label end should be used for the distilled wate incubations. Slotted plastic trays contain the other solutions, whic should completely cover the slide.

1. Histoclear
2. Histoclear
3. 100% isopropanol
4. 70 % isopropanol
5. Distilled water
6. Distilled water
7. Triton X100 solution
8. TBS

IV. Assignment of Student Groups:

This laboratory was designed for eight students (or pairs o students) with each student or student pair preparing a single slide

Group 1 - Five students (or 5 pairs of students) will use the antibody to alpha tubulin

Group 2 - One student will use BSA solution in place of the tubulin antibody. This serves as a negative control, which is needed in order to assess the background staining by the tubulin antibody.

Group 3 - Two students will stain their slides with hematoxylin and eosin in order to better see the fine structures of the trachea and esophagus.

At the end of this exercise, students will exchange slides so that they will see the results of all groups.

V. Safety Considerations

Histoclear II is manufactured from 100% food grade materials and has been reported to be nontoxic and completely biodegradable by the manufacturer. The peroxidase substrate solution contains hydrogen peroxide and TMB. TMB has been reported to be a noncarcinogenic substrate of peroxidase - See: V. Holland, Tetrahedron, 30, 3299, (1974)

IND 26 Localizing Tubulin by Immunohistochemistry

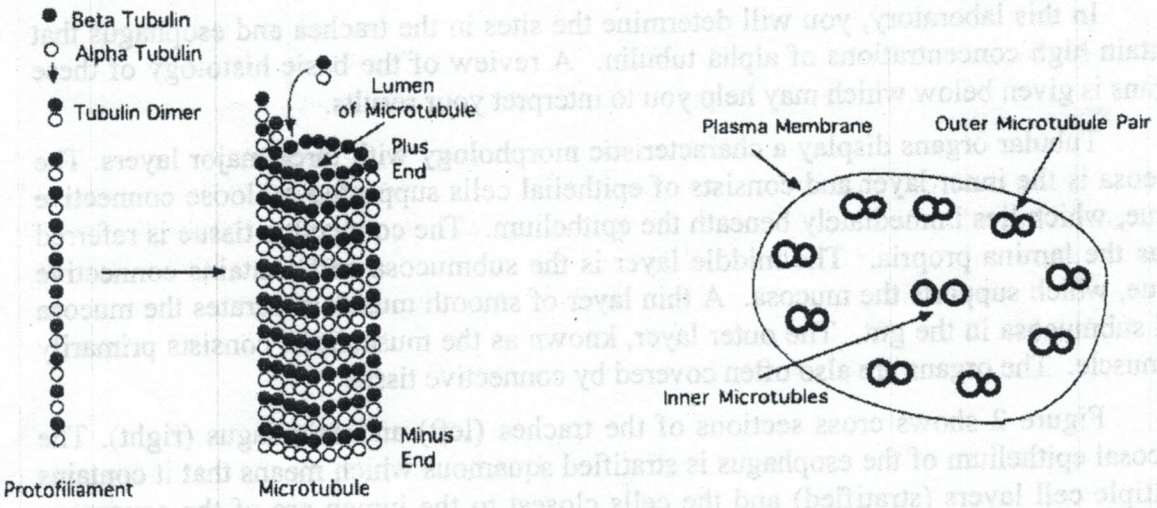
Background Information

1. Microtubules and Tubulin

The cytoskeleton is the scaffolding of the cell consisting of a dynamic network of protein filaments. The cytoskeleton serves to maintain cell shape and also plays important roles in cell motion, cell division and the transport of vesicles and organelles within cells. The three types of proteins filaments that comprise the cytoskeleton are the microtubules, the intermediate filaments and the actin filaments.

Microtubules are hollow cylinders made up of tubulin polymers. Tubulin is itself a dimer containing two globular protein subunits called alpha and beta tubulin. To form a microtubule, tubulin molecules stack together to form protofilaments. Thirteen parallel protofilaments form a single microtubule as shown in Figure 1. Tubulin molecules assemble onto one end (the + end) of a microtubule while the other end usually forms a stationary anchor. The process repeats rapidly resulting in the polymerization and thus elongation of the microtubule toward the + end. Depolymerization of the microtubule into free tubulin then occurs which causes the microtubule to shorten.

Figure 1. Tubulin and Microtubules



Left-Tubulin contains protein subunits called alpha and beta tubulin. To form a microtubule, tubulin molecules stack together to form protofilaments. Thirteen parallel protofilaments form a single microtubule.

Right-Cross section of a cilium or flagellum showing the 9+2 arrangement of microtubules. The position of dynein is not shown in the figure.

Copyrighted by John N. Anderson, 2007

Microtubules are structural supporters in the cytoplasm and serve as substrates along which motor proteins can move. They are also capable of growing and shrinking and this dynamic feature is responsible for a variety of cellular movements. For example, microtubules are major components of the mitotic spindle, which is used by cells to segregate their chromosomes during cell divisions.

Microtubules are also major components of cilia and flagella, which are tail like projections that are covered by plasma membrane and extend outwards from the cell. Motile cilia are used for locomotion and food gathering by some protozoa (ciliates) and are found in the lining of the trachea (windpipe), where their wave like motion propels mucus, dust and other foreign matter out of the lungs. Flagella serve to propel spermatozoa and several types of protozoa. Flagella are similar to cilia internally, but are usually much longer and fewer in number per cell.

Cilia and flagella are composed of a central cylinder, which contains a common arrangement of microtubules. The arrangement consists of nine outer pairs of microtubules and an additional two microtubules in the center as seen on right of Figure 1. The central core also contains dynein, which is a microtubule-associated protein (MAP). The dynein is responsible for the sliding of the microtubules past one another, which serves to bend the cilium or flagellum. The bending occurs in the form of a wave that begins at the base of the cilium or flagellum and proceeds to the tip.

2. Trachea and Esophagus

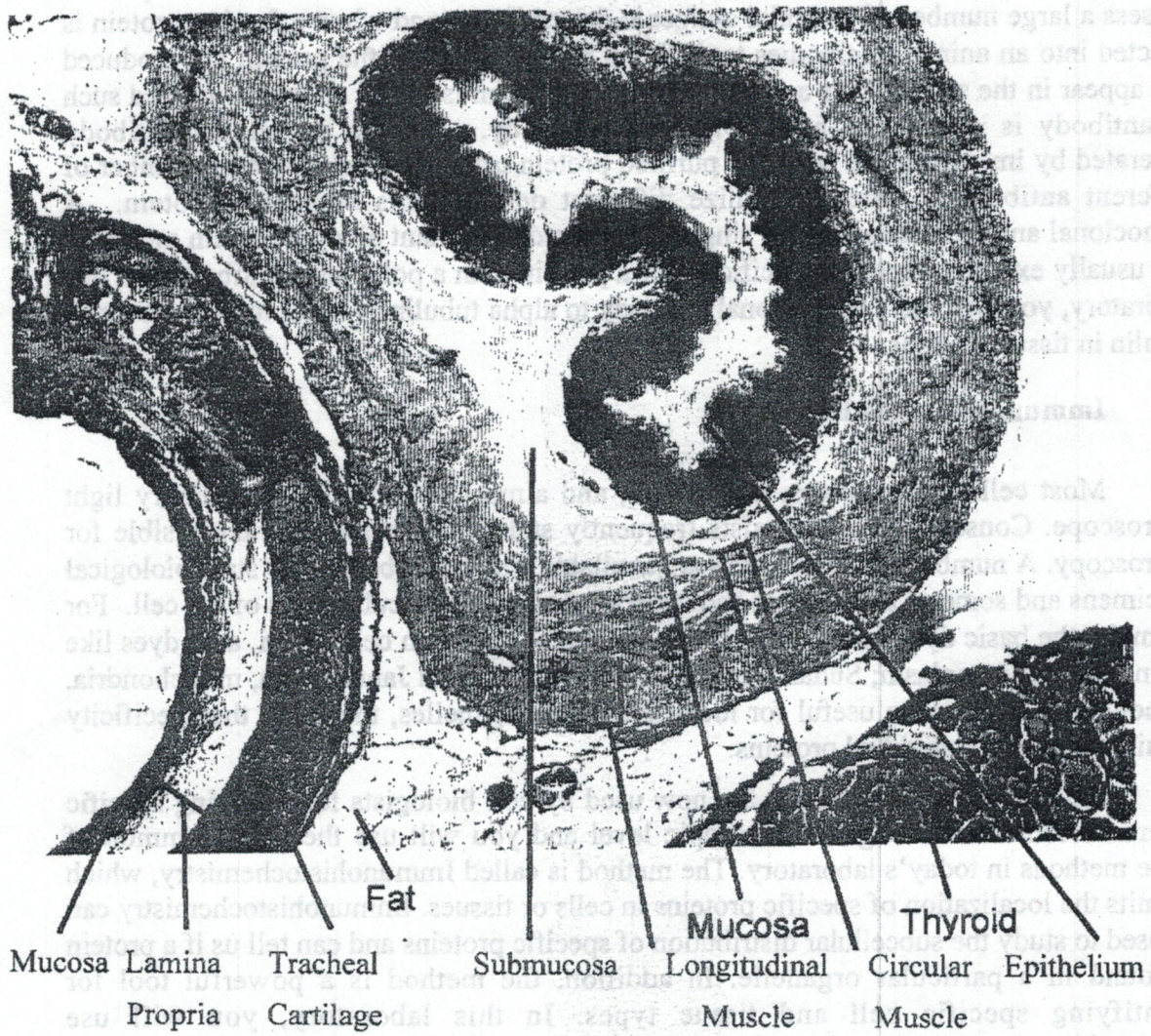
In this laboratory, you will determine the sites in the trachea and esophagus that contain high concentrations of alpha tubulin. A review of the basic histology of these organs is given below which may help you to interpret your results.

Tubular organs display a characteristic morphology with three major layers. The mucosa is the inner layer and consists of epithelial cells supported by loose connective tissue, which lies immediately beneath the epithelium. The connective tissue is referred to as the lamina propria. The middle layer is the submucosa and contains connective tissue, which supports the mucosa. A thin layer of smooth muscle separates the mucosa and submucosa in the gut. The outer layer, known as the muscularis, consists primarily of muscle. The organs are also often covered by connective tissue.

Figure 2 shows cross sections of the trachea (left) and esophagus (right). The mucosal epithelium of the esophagus is stratified squamous which means that it contains multiple cell layers (stratified) and the cells closest to the lumen are of the squamous (flat) type. The multiple layers are an adaptation to its role in forcing foodstuff toward the stomach, thus its abrasion resistance. The meshwork of connective tissue fibers, vessels and nerves underlying and physiologically supporting the mucosa is the submucosa. The esophagus pushes food by serially contracting its strong circular and longitudinal muscles of the muscularis.

The trachea lies in close proximity and ventral to the esophagus. Its basic architecture resembles that of the esophagus with modifications reflecting its role of gas delivery to and from the bronchi and lungs. The mucosa is comprised of ciliated, columnar epithelial cells (called pseudo stratified because the various cell nuclei present the illusion of stratification). There is an unusually thick, elastic connective tissue lamina propria between the mucosa and the tracheal cartilages. The tracheal cartilages are skeletal elements composed of chondrocytes (cartilage cells), which are imbedded in an amorphous matrix of their secretion. The tracheal cartilages hold open the tracheal lumen during inhalation and exhalation, serving the same function as the coil spring or corrugations in the wall of a vacuum-cleaner hose.

Figure 2. Cross Sections of Trachea (left) and Esophagus (right).



3. Antibodies

Antibodies form the basis of the method that you will use in today's laboratory and a review of these powerful tools seems in order. The macromolecules that elicit antibody production are called antigens and are most often proteins. Although antigens are normally components of foreign organisms, purified proteins will serve as antigens in that they will stimulate the formation of antibodies when injected into a suitable test animal such as rabbit. Each antigen possesses features that are recognized by the antibody and these features constitute the antigenic determinants or epitopes. An antigenic determinant recognized by an antibody molecule may be a unique shape or a sequence of about 7 to 10 amino acid residues on the protein molecule. It follows that each protein possess a large number of potential antigenic determinants and when a foreign protein is injected into an animal, antibodies to different determinants on the protein are produced and appear in the serum. This antibody containing serum is called an antiserum and such an antibody is referred to as a polyclonal antibody. Thus, a polyclonal antibody generated by immunization with one purified protein usually contains a large number of different antibodies, which recognize different determinants along the protein. A monoclonal antibody recognizes a single antigenic determinant along a protein molecule and usually exhibits a greater specificity for a protein than a polyclonal antibody. In this laboratory, you will use a monoclonal antibody to alpha tubulin in order to localize alpha tubulin in tissue sections.

4. Immunohistochemistry

Most cells are colorless, translucent, and almost invisible in an ordinary light microscope. Consequently, biologists frequently stain cells to render them visible for microscopy. A number of organic dyes are available which can be used to stain biological specimens and some of these stains show a preference for particular parts of the cell. For example, the basic dyes methylene blue and hematoxylin stain cell nuclei, acid dyes like eosin stain the cytoplasm; Sudan black stains fat droplets and Janus green, mitochondria. Although these dyes are useful for identifying cell organelles, they lack the specificity required to detect individual proteins.

A variety of new methods are now used by cell biologists for detecting specific macromolecules at the light microscopic level and you will use the most common of these methods in today's laboratory. The method is called Immunohistochemistry, which permits the localization of specific proteins in cells or tissues. Immunohistochemistry can be used to study the subcellular distribution of specific proteins and can tell us if a protein is found in a particular organelle. In addition, the method is a powerful tool for identifying specific cell and tissue types. In this laboratory, you will use immunohistochemistry in order to localize alpha tubulin in the esophagus and trachea.

Materials

Materials Provided

Trachea/Esophagus Slides
*Isopropanol or ethyl alcohol (100%, 70 % and 30 %)
Clearing Agent (Histoclear II)
Mounting Medium (Histomount)
Coverslips
Slide Staining Dishes
Large Transfer Pipets
Small Transfer Pipets
Glass Rods
*TBS (Tris Buffer Saline)- The working solution contains 0.15 M NaCl and 10 mM Tris, pH 8.0.
Hematoxylin
Eosin
Alpha tubulin antibody- The tubulin antibody is a mouse monoclonal antibody
Second antibody-This antibody binds to the mouse tubulin antibody. It is complexed with peroxidase, which catalyzes a color producing reaction. The second antibody is an Anti-mouse IgG-peroxidase antibody produced in goat.
Peroxidase Substrate
BSA Blocking Solution -The BSA blocking solution contains 3% BSA (bovine serum albumin), 0.15 M NaCl and 10 mM Tris, pH 8.0 and the antibodies are diluted in BSA Blocking solution.
*Triton X 100- The working solution contains 0.5 % Triton X100 and 10 mM Tris, pH 8.0

*Prepared as described in the Instructor Guide

Materials Needed but not Provided

Beakers
Pin or Needle probes
Newspaper or paper towels
Forceps
High Quality distilled water

Procedure

Theory and Description:

The slides that you will use in today's laboratory have been preprocessed and are ready for immunohistochemical analysis but it is important that you know how they were prepared and how to handle them. Small pieces of tissue were exposed to large volumes of acids, alcohols and/or aldehydes, which quickly denatures and inactivates proteins in the living cells. This fixed them, thus preserving their structural integrity. These samples were then dehydrated through a graded alcohol series to remove the fixatives and to condition them for infiltration of the tissue by liquid paraffin. When solidified, the paraffin allows easy manipulation of the tissue and gives it structural support. Once this material completely penetrates the tissue, the sample is positioned in a large volume of the same material and allowed to solidify, a process called embedding. By appropriately positioning the tissue, its surrounding block can then be attached to the working arm of a microtome. This device allows the operator to precisely section (cut) thin (usually 2-10 micra) slices of tissue and its supporting block. Selected slices are then mounted on a glass slide coated with an adhesive (such as albumin), which anchors the section to the slide, thus stabilizing it for storage or shipping.

Infiltration/embedding media prevent staining with antibodies so they must be removed. This is accomplished by soaking the slide in a clearing agent (Histoclear II), which dissolves the infiltration/embedding media. The tissue is then rehydrated by passing the slide through a series of alcohol solutions ending with water, which reverse the dehydration. The slide is then treated with the detergent Triton X 100 that ruptures membranes thereby increasing the accessibility of antigens to antibodies and then with the BSA blocking solution. The BSA (bovine serum albumin) in the solution and in the antibody solutions binds to nonspecific sites thus reducing nonspecific antibody binding. The tissues are then exposed to the tubulin antibody, which binds to the tubulin and then to the secondary antibody, which binds to the first antibody. The second antibody is complexed with peroxidase, which catalyzes a color producing reaction. Finally, the tissue is incubated with the peroxidase substrate solution and the peroxidase activity associated with the second antibody will convert the colorless substrate into a blue product, which serves to "stain" the area containing the alpha tubulin.

Notes on the procedure:

Work on newsprint or paper towels to absorb slide runoff or spilled liquids. The instructor or one student in the class should arrange the 8 solutions in step 1 (below) in the sequence indicated below. Beakers or similar containers holding sufficient water to cover a slide submerged from the label end should be used for the distilled water incubations. Slotted plastic trays contain the other solutions, which should completely cover the slide.

Group Assignments:

This procedure was designed for eight students or (or pairs of students) with each student preparing a single slide. At this time, you should pick one of the following treatment groups:

Group 1 -Five students will use the antibody to alpha tubulin

Group 2-One student will use BSA solution in place of the tubulin antibody. This serves as a negative control, which is needed in order to assess the background staining by the tubulin antibody.

Group 3-Two students will stain their slides with hematoxylin and eosin in order to better see the fine structures of the trachea and esophagus.

At the end of this exercise, each student will view slides from each of the three groups so that you will view your slide as well as the slides of your classmates.

Step by Step Instructions:

Groups 1,2 and 3.

1. Each student should place one slide in the following solutions for the indicated times. Use forceps to handle slides between solution stations. As you move slides from one station to the next, you should briefly touch its edge to the paper, thus draining off excess surface solution. But DO NOT allow the slide to dry at any time. Following transfer to each new solution, close the lid of the tray and gently rock the tray back and forth a few times.

    1. Histoclear-3-5minues
    2. Histoclear-3-5 minutes
    3. 100% Isopropanol 1-2 minutes
    4. 70 % isopropanol 1-2 minutes
    5. Distilled water-2-3 minutes
    6. Distilled water-2-3 minutes
    7. Triton X100 solution- 5-7 minutes
    8. TBS 2-3 –minutes

Groups 1 and 2-Antibody Staining

1. Place the slide, labeled side up, on the bench in front of you. Carefully add 100ul of the BSA blocking solution to cover the tissue on the slide. During the process make sure that you do not touch the tissue with the pipette tip. A microliter pipetor should be used to add the solution to the slide. If a microliter pipette is not available, use a SMALL transfer pipet to dispense about 100 ul. Approximately 3-4 drops is equal to 100 ul. Return any solution in the pipet to the original stock tube.

2. After 5 minutes, drain the slide by standing it on end and then place it back on the bench in front of you. Group 1 should then place 100 ul of the tubulin antibody to the section. Group 2 should place 100ul of the BSA blocking solution to their slide.

3. After 20 minutes, rinse the slide with TBS. To rinse the slide, hold it on the edge with the label at a 90° angle over an empty beaker. Draw a few mls of TBS into a large transfer pipet, slowly expel the TBS onto the top of the slide near the labeled edge, and permit the TBS to flow down the slide over the tissue. Repeat this process four times.

4. Drain the slide well by standing it on end and then place it back on he bench in front of you. Carefully add 100ul of the second antibody to cover the tissue on the slide.

5. After 20 minutes, rinse the slide with TBS as described above in step 4.

6. Rinse the slide four times with distilled water, drain it as described above and then place it back on the bench in front of you.

7. Place 100 ul of the peroxidase substrate onto the tissue.

8. After 10 minutes, rinse the slide three times with water and place it in a slotted tray containing 30 % isopropanol in order to start the dehydration process.

9. After 3-4 minutes, transfer the slide to slotted tray that contains 70% isopropanol.

10. After 3-4 minutes, transfer the slide to slotted tray that contains 100% isopropanol.

11. After 3-4 minutes, air-dry the slide for a few minutes and then place it labeled side up, on the bench in front of you.

12. Using a glass rod, place a large drop of mounting medium on the stained tissue. Gently lower the coverslip into the mounting medium and over the tissue. Try to prevent trapping air bubbles under the coverslip. A pin or needle-probe is ideal for this. Center the coverslip over the tissue.

13. The slides can be carefully viewed under low power of your microscope at this time. During this time, always hold the slide in a horizontal position. However, the slides should NOT be viewed under oil immersion until the next laboratory session since the mounting medium has not hardened. Store the slide face-up in a clean, undisturbed place in the dark and let it cure. After a few days the mounting medium hardens and your slides are ready to examine in detail. Always handle a slide by the label end and avoid touching

the coverglass. Soiled/smudged coverglasses can be cleaned by gently wiping with 70% alcohol.

Group 3- Staining with Hematoxylin and Eosin

1. Place the slide, labeled side up, on the bench in front of you. Carefully add 100ul of the Hematoxylin solution to cover the tissue on the slide. During the process make sure that you do not touch the tissue with the pipette tip. A microliter pipetor should be used to add the solution to the slide. If a microliter pipette is not available, use a SMALL transfer pipet to dispense about 100 ul. Approximately 3-4 drops is equal to 100 ul. Return any solution in the pipet to the original stock tube.

2. After 5 minutes, drain the slide by standing it on end and then rinse the slide with water. To rinse the slide, hold it on the edge with the label at a 90° angle over an empty beaker. Draw a few mls of water into a large transfer pipet, slowly expel the water onto the slide near the labeled edge, and permit the water to flow down the slide over the tissue. Repeat this process eight times.

3. Drain the slide well by standing it on end and then wash the slide three times as described above with 70% alcohol.

4. Place the slide, labeled side up, on the bench in front of you. Carefully add 100ul of the eosin to cover the tissue on the slide.

5. After 30 seconds, rinse the slide four times with 100 % alcohol, drain it as described above.

6. Place the slide in a tray containing Histoclear.

7. After 1 minute, transfer the slide to a second tray containing Histoclear.

8. After 3-5 minutes in the the last Histoclear, rest the wet slide on the paper face-up. Using a glass rod, place a large drop of mounting medium near the stained tissue. Dip a coverslip in the last Histoclear solution and place its EDGE on the slide NEAR the drop. GENTLY lower the wet coverslip into the mounting medium and over the tissue to prevent trapping air bubbles. A pin or needle-probe is ideal for this. Center the coverslip over the tissue, then tilt the slide up on its LONG EDGE to drain off excess Histoclear.

9. The slides can be carefully viewed under low power of your microscope at this time. During this time, always hold the slide in a horizontal position. However, the slides should NOT be viewed under oil immersion until the next laboratory session since the mounting medium has not hardened. Store the slide face-up in a clean, undisturbed place in the dark and let it cure. After a few days the mounting medium hardens and your slides are ready to examine in detail. Always handle a slide by the label end and avoid touching the coverglass. Soiled/smudged coverglasses can be cleaned by gently wiping with 70% alcohol.

Microscopy and Data Analysis
(Groups 1-3)

1. Group 3- Examine your slides with the low and medium power objectives of your microscope. First identify the trachea and esophagus and then all tissue that are labeled in Figure 2.

2. Groups 1 and 2- Examine the regions of the tissues that were stained blue with the low and medium power objectives of your microscope and try to identify the regions in both trachea and esophagus that contain the highest amounts of alpha tubulin. Refer to Figures 2 for your histological description.

3. Groups 1-3-Exchange your slide with those of your classmates and note differences that that are seen between the 8 slides.

Study Question

Describe a procedure that you would use to localize the protein dynein in tissue sections of esophagus and trachea. Indicate the results that you would expect from such an analysis.