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DEPARTMENT OF BIOTECHNOLOGY

M.TECH

IMMUNOTECHNOLOGY LABORATORY

(481172L2)

HOD

IMMUNOTECHNOLOGY LAB MANUAL

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GENERAL LABORATORY PROCEDURES, EQUIPMENT USE, AND SAFETY CONSIDERATIONS

CHEMICALS

A number of chemicals used in any molecular biology laboratory are hazardous. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. The following chemicals are particularly noteworthy:

- Phenol - can cause severe burns
- Acrylamide - potential neurotoxin
- Ethidium bromide - carcinogen
- Formaldehyde, Acetonitrile, Chloroform are potentially harmful

These chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipette them. If you accidentally splash any of these chemicals on your skin, **immediately** rinse the area thoroughly with water and inform the instructor. Discard the waste in appropriate containers.

ULTRAVIOLET Light

Exposure to ultraviolet light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, **always wear appropriate eye protection when using UV lamps.**

ELECTRICITY

The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

GLASSWARE AND PLASTIC WARE .

Glass and plastic ware used for molecular biology must be scrupulously clean. Dirty test tubes, bacterial contamination and traces of detergent can inhibit reactions or degrade nucleic acid.

Glassware should be rinsed with distilled water and autoclaved or baked at 150 degrees C for 1 hour. Make sure that the tubes you are using are resistant to the chemicals used in your experiment. Micro pipet tips and microfuge tubes should be autoclaved before use.

GENERAL HOUSEKEEPING

All common areas should be kept free of clutter and all dirty dishes, electrophoresis equipment, etc should be dealt with appropriately. Since you have only a limited amount of space to call your own, it is to your advantage to keep your own area clean. Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. **must be labeled.** In order to limit confusion, each person should use his initials or other unique designation for labeling plates, etc. Unlabeled material found in the refrigerators, incubators, or freezers may be destroyed. Always mark the backs of the plates with your initials, the date, and relevant experimental data, e.g. strain numbers.

DECONTAMINATION of Ethidium Bromide Spills

EtBr spills can be decontaminated with a solution of 20 ml of hypophosphorus acid (50%) added to a solution of 4.2 g of sodium nitrate in 300 ml water. Prepare fresh solution the day of use in a fume hood. Wear rubber gloves, lab coat, and safety glasses. Turn off electrical equipment before decontamination.

- Soak paper towel in decontamination solution, place on contaminated surface, and scrub.
- Scrub five more times with paper towels soaked in water, using fresh towel each time.
- Place all towels in a container and soak in fresh decontamination solution for one hour.
- Test squeezings from final towel scrub and mixture for fluorescence; repeat procedure
  with fresh decontamination solution if fluorescence is present.

- Neutralize with sodium bicarbonate and discard as nonhazardous aqueous waste.
- This procedure has been validated for EtBr-contaminated stainless steel, Formica, glass, vinyl floor tile surfaces, and filters of transilluminators.

Alcohol solutions that characterize as hazardous wastes are prohibited from discharge to the sewer.

The waste alcohol must be diluted with plenty of water inorder to reduce the concentration of the alcohol concentration before its discharge into the sewer.

DISPOSAL OF AGAR/LB PLATES

Any uncontaminated, solidified agar or agarose should be discarded in the trash, not in the sink, and the bottles rinsed well.Any media that becomes contaminated should be promptly autoclaved before discarding it. Petri dishes and other biological waste should be discarded in Biohazard containers which will be autoclaved prior to disposal.

CLEANLINESS OF THE LABORATORY

The gel apparatus should be washed immediately after use. The glass plates must be rinsed with plenty of tap water, ethanol and finally with distilled water, wipe it with tissue paper. Beakers, measuring cylinders and all other glass wares should be rinsed with distilled water before and after use. The working area must be kept clean without any spillage, broken glass wares before and after the use.

IDENTIFICATION OF CELLS IN BLOOD SMEAR

EXPT NO. 1
DATE :
AIM

To identify different WBC cells present in the blood smear

MATERIALS REQUIRED

Blood sample, microscopic slides, leishmann's stain, light microscope and double distilled water.

PROCEDURE

- A drop of blood is placed over the clean glass slide at the centre and spread the blood to form a smear.
- Air dry the blood smear and label properly
- Apply leischmann's over the smear and allow for couple of minutes, then add equal volumes of double distilled water and mix properly.
- Keep the slide aside for 10-20 minutes and wash the slide in running water and air dry the smear.
- After air drying, observe the blood smear under light microscope.

RESULT

The following cells were identified in the blood smear using light microscope

IDENTIFICATION OF BLOOD GROUPS

EXPT NO. 2
DATE :
AIM

To demonstrate antigen-antibody reaction by Hemeagglutination reaction.

PRINCIPLE

Blood group classification have extreme importance in medical field. ABO blood groups were discovered by Carl Landsteiner. The system classifies human blood into four main groups based on the presence or absence of the cell surface antigens.

- If the red cells have only antigen A at its surface, the blood type is 'A' where the plasma contains Anti-B antibodies which clumps the cells.
- If the red cells have only Antigen B, the blood is type 'B' where the plasma contains Anti-A antibodies, which clumps cells having antigen A.
- If the cells have both antigen A and antigen B, the plasma contains neither Anti-A or Anti-B.
- Rh blood types form the second major blood group system. Rh factor agglutinate with Anti-Rh antibody. This reaction can produce serious illness or death. When the blood agglutinates with Anti-Rh antibody, it indicates the person is Rh positive and if there is no agglutination, it indicates the person is Rh negative.

ABO blood grouping has tremendous application in the field of blood transfusion, tissue typing, graft transfer and other medical ailments.

MATERIALS REQUIRED

Blood sample, antiserum (anti-A, anti-B, anti-D), glass slide, lancet or needle, rectified spirit and cotton.

PROCEDURE

- Label three glass slides as A,B, and D .
- Add one drop of blood to each labeled areas.
- Add a drop of antisera-A to labeled A, a drop of antisera-B to labeled B and a drop of antisera D to labeled D.
- Mix the antisera with blood slowly and properly using the edges
- After mixing, observe for agglutination if any.

OBSERVATION

- Aggregates of blood sample is observed in the labeled glass slide.

INTERPRETATION

- Agglutination of blood with antisera occurs. If agglutination occurs at glass slide A, it is A blood group.

- If agglutination occurs at glass slide B, it is B blood group.
- If there is no agglutination in glass slide A and B, it indicates O blood group.
- Agglutination at glass slide D indicates the presence of Rh factor.

RESULT

The blood sample was analyzed and the blood group is found to be_____

IMMUNO ELECTROPHORESIS

EXPT NO. 3
DATE :
PRINCIPLE:

Immunoelectrophoresis consists of two methods that are combined, namely agar-gel electrophoresis and immunodiffusion. The agar plate has a longitudinal trench cut which accommodates the antiserum after electrophoresis and a central well or wells to accommodate test material. The serum sample is placed in the well or wells and electrophoresis is carried out for 1-2h at a field strength of 5-9 V/cm. The protein components separate according to their charge, molecular weight and shape but unlike normal electrophoresis, the bands are not stained for proteins. Instead antiserum is pipetted into the longitudinal trough and the plate is incubated in humid chamber for overnight. Diffusion of the antiserum and protein occurs and, where they meet, precipitin arcs are formed that give rise to a characteristic pattern.

MATERIALS:

1. 0.07M Baribiturate buffer; pH 8.6 (Dissolve 2.58g of diethylbarbituric acid and 14.42g of sodium diethylbarbiturate in water and make up to 1litre).
2. Agarose (medium EEO) solution (1% w/v in the barbiturate buffer: heat the agarose at 90^0C until all the lumps are dissolved and clear solution is obtained and pour on to a slide when it is cooled to say 55^0C. Allow the agarose to solidify.)
3. Glass slides (7.5 cm x 5cm).
4. Incubator at 37^0C
5. Horizontal electrophoresis apparatus
6. Power supply
7. Cords
8. Gel puncher
9. Shandon cutting device
10. Bromophenol blue
11. Saline (0.9%w/v NaCl; 0.1w/v Na azide)
12. Antigen
13. Rabbit antiserum or peak fraction of the first peak in the previous experiment.
14. 0.25% coomassiee brilliant blueR_{250} prepared in 4:1:5 (v/v/v) in ethanol: acetic acid: distilled water.
15. Destain solution (4:1:5 (v/v/v) in ethanol: acetic acid: distilled water).

METHOD:

- Heat glass plates in an oven at 100^0C for about 30 minutes then remove them and place on a level surface.
- Carefully pipette about 8 ml of the hot agarose solution on to the surface of the slides.
- The molten agarose will set in a uniform layer and the surface tension will hold the agarose at the edges. Allow the agarose to solidify.

- Punch the gel with one well of relatively bigger size and with the help of Shandon cutting device give 4.5 cm long trough of 1mm diameter. With the help a of sharp needle remove the agarose from the well as well as from the trough.
- The antigen is mixed with 0.025% bromophenol blue, the tracking dye. Fill the electrophoresis chamber with 0.07M barbiturate buffer and place the slide in the chamber and connect the slide with wigs prepared from Whatman No 3 filter papers on either side and the wigs must dip into the buffer solution.
- Apply the potential difference of 5-9 V/cm for about 1-2h or until the tracking dye reaches the 2.0 -2.5 cm from the well.
- Remove the slide from the electrophoresis chamber and add of the antiserum or purified IgG place the slide in the moist chamber and incubate for overnight at 37^0C and observe the precipitin line formation on the following day.

OBSERVATION

- The appearance of the precipitin line is noticed in the presence of specific antibody.

INTERPRETATION

- Immunoelectrophoresis is a diagnostic tool for qualitative and quantitative determination of biological samples.
- The specificity of the antigen can be ascertained to the specific antibody based on the pattern of precipitin arc.

RESULT

Thus the specific of the antigen towards the specific antibody is ascertained.

PURIFICATION OF IMMUNOGLOBULIN (IGG FROM EGG YOLK)

EXPT NO. 4
DATE :
AIM

To purify chicken immunoglobulin G from egg yolk.

Principle

Chicken IgG is also known as IgY. It is the major serum antibody. It is transported to the egg similar to placental transfer of IgG in mammals. This experiment works on the mechanism of precipitation of organic polymers. Organic polymers dicrease the dielectric constant of solution and hence its solvating power. Thus the solubility of protein can occur due to electrostatic attraction. Precipitation occurs when pH is close to isoelectric point of the protein.

REQUIREMENTS

Chicken egg, glasswares, magnetic stirrer/ magnetic pellet, sterile syringe, gauze cloth, precipitation solution, PBS, SDS-PAGE reagents and solutions.

PROCEDURE

- The egg yolk is initially warmed to room temperature.
- The egg is carefully cracked under sterile conditions and its contents are poured in a sterile petri dish, without disturbing the yolk.
- The egg white was separated completely using syringe (without needle)
- The egg yolk was poured in beaker and its weight was measured.
- The yolk was stirred at room temperature with slow addition of 3 volumes of precipitation solution.
- Strring was continued to precipitate the lipids.
- This solution was centrifuged at 10000rpm for 15 minutes.
- The supernatant was collected by filtering through fine gauze cloth.
- The volume of supernatant was measured and 1/3 volume of ammonium sulphate was added to it.
- The mixture was stirred for 10 minutes to precipitate IgG.
- The solution was centrifuged at 10000rpm for 15min at 4C.
- Supernatant was discarded and pellet was washed with. PBS and finally suspended in PBS.
- SDS-PAGE analysis of purified IgG was carried out.

RESULT

The immunoglobulin-Y (IgY) was purified from the chicken egg yolk and fractionated using SDS-PAGE.

SINGLE RADIAL IMMUNO DIFFUSION (SRID)

EXPT NO. 5
DATE :
AIM

To quantify the amount of antigen present in test sample by single radial immuno diffusion.

PRINCIPLE

Radial immuno-diffusion is a method for determining the concentration of an antigen. The antigen sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an anti-serum. The area of the precipitin ring that forms around the well in the region of equivalence is proportional to the concentration of the antigen.

The antigen-antibody precipitation is made more sensitive than in double immuno diffusion by the incorporation of the antiserum in the agarose solution before the gel is made. Thus the antibody remains uniformly distributed throughout the gel. Antigen is then allowed to diffuse from wells cut in the gel. This is an example of single immuno diffusion.

Initially as the antigen diffuses out of the well, the antigen concentration is relatively high and soluble antigen-antibody adducts are formed. However, as antigen diffuses farther from the well, the antigen-antibody complex reacts with more amount of antibody resulting in a lattice that precipitates to form a precipitin ring. The concentration of antigen is directly proportional to the diameter of the precipitin ring.

REQUIREMENTS

Antigen, antibody, test antigen. Agarose , gel punch, class slide, saline, template. Titer plates, micropipette, glasswares etc.,

PROCEDURE

- 1% agarose was prepared in saline and heated to dissolve the agarose.
- At optimum temperature (~40C), antibody of desired volume is added to agarose 3-4mm of agarose gel is prepared on microscopic slide.
- The slides were stored at 4C for a short time.
- After agarose has hardened sufficiently, two sets of wells, 3 on top and3 on bottom was cut using gel punch with the help of template.
- The antigen was diluted two fold in micro titer plate.
- Antigen is serially diluted in normal saline to obtain 1:2, 1:4,1:8,1:16 dilutions.
- 10ul of undiluted antigen was added to the first well of the agarose gel.
- 10ul of serially diluted antigen was added to 2^{nd}, 3^{rd}, 4^{th} and 5^{th} wells.
- 10ul of sample antigen was added to the 6^{th} well.
- After filling the wells, the slide was kept in a flat bottom container whose interior was kept moist by using damp cotton.
- The slides were placed at room temperature for 18-24 hours.
- Diameter of precipitin ring was measured and calibration curve was plotted.

RESULT

The antibody titration at various dilution was carried out and the concentration of the test antigen was found to be

DOUBLE DIFFUSION (OUTCHERLONY METHOD)

EXPT NO. 6
DATE :
AIM

To quantitate and characterize antigen and antibodies by ouchterlony double diffusion

PRINCIPLE

Immunodiffusion in gel encompasses a variety of techniques, which are useful for the analysis of antigen and antibodies. One such method is ouchterlony double diffusion. Both qualitative and quantitative information can be obtained from double diffusion assays. This techniques is used for comparing different Ag preparations.

In the ouchterlony method, both antigen and antibody diffuse radially from wells towards each other, thereby establishing a concentration gradient. As equivalence is reached, a visible live of precipitation is formed. This simple technique is an effective quatitative tool for determining the relationship between antigens and the number of different antigen-antibody systems present. The pattern of the precipitin lines that form, where two different antigen preparations are placed in adjacent wells indicates whether they share epitopes. The pattern of lines that form, can be interpreted to determine whether, the antigens are same or different.

REQUIREMENTS

Microscopic slide, template, gel punch, saline, agarose, antigens and antibodies, test serum, glasswares etc.,

PROCEDURE

- 1% agarose was prepared in normal saline and heated to polymerize the agarose
- 3-4mm thick agarose gel was prepared on glass slide
- The slides were stored at 4C for a short time.
- After agarose has hardened sufficiently, two sets of wells were cut using gel punch with the help of template.
- Antibody and the test serum was diluted to two fold and used.
- In the first set well, antigen was added in central well and diluted antibody was added in surrounding wells (1:2 to1:16 dilutions)
- In second set, antigen was added to central well and diluted test serum was added in surrounding wells.
- After filling the wells, the slide was kept in a flat bottom container whose interior was kept moist by using damp cotton.
- The slides were placed at room temperature for 18-24 hours.

RESULT

The antibody titration at various dilution was carried out and the variation in the precipitin arc was observed.

HAEMEAGGLUTINATION

EXPT. NO. 7

DATE .

AIM

To perform agglutination reaction

PRINCIPLE

Haemagglutination is a convenient and simple technique. Some viruses have the property of adsorbing to red blood cells through the receptor sites on their surface resulting to form haemagglutination, i.e., clumping of red blood cells.

MATERIALS REQUIRED

1%Chicken RBC, micropipettes, glass wares, distilled water.

PROCEDURE

Preparation of virus (HA antigen)

- Dissolve the virus in freeze dried vial with 1ml of diluent and mix it properly. Store at 4˚C.

Preparation of 1% chicken erythrocytes (CRBC)

- Collect chicken blood from healthy chicken in Alsever's solution and carefully centrifuge at 2000 rpm for 5 minutes to pack the red blood cells.
- Discard the supernatant. Wash the CRBC by adding 10ml of saline and mix it well.
- Again centrifuge at 2000 rpm for 5 minutes and discard the supernatant, repeat the procedure for three times.
- After discarding the supernatant, the sedimented RBC's are reconstituted to a final concentration of 1% in normal saline.
- Add 25µl of normal saline to all the wells in 'A' arrow of a V bottom 96 well microtitre plate.
- Add 25µl of antigen to the first well and carry out serial dilution.
- The last well acts as control (RBC control)
- Add 25µl pf normal saline to all the wells.
- Add 50µl of 1% chicken RBC to all wells of the microtitre plate.
- Gently tap the microtitre plate for efficient mixing.
- Wrap it with aluminium foil and keep the microtitre plate at 4˚C for the settling of RBC's .

RESULT

The titre value of the virus can be ascertained and the titre value is found to be_____

TESTING FOR TYPHOID ANTIGENS BY WIDAL TEST

EXPT.NO. 8

DATE.

AIM

To diagnose the suspected case of Enteric fever serologically

PRINCIPLE

Widal test is serological method used in the diagnosis of Enteric fever which is caused by the organisms of the genus salmonella. They are *salmonella typhi, Salmonella Paratyphi* A,B,C,D*,* etc.

Enteric fever due to *S.typhi* and *S.Paratyphi* A,B,C and D are common in India. Antigens specifically prepared fromthis orgaism are used in the agglutination test to detect the presence of antibodies in patient sera which are elucidated in response to infection by these bacteria.

The organisms causing enteric fever possess two major antigens namely somatic 'O' antigen and flagellar "H' antigen along with another 'VI" surface antigen. During infection, antibodies are produced in patients sera against these 'O', 'H' *S.typhi* , '*A(H)'* and '*B(H)' S.paratyphi* antigens.

MATERIALS REQUIRED

Six circle glass slides, disposable mixing sticks, positive patient serum, micropipette.

PROCEDURE (Rapid Slide Test-Screening test)

- Take a properly cleaned glass slide
- Add a drop of undiluted test serum in the corresponding reaction circles and a drop of positive and negative control serum in the circles marked positive control and negative control.
- Add a drop of antigen O,H, A(H) amd B(H) in respective circles.
- Add any one of ht 'H' antigen [H,A(H), or B(H)] in the positive and negative control.
- Mix the contents of each circle with separate disposable mixing sticks.
- Keep the slide for one minute and then observe the visible agglutinins.

RESULT

SEPARATION OF T AND B LYMPHOCYTES FROM HUMAN PERIPHERAL BLOOD(T-Cell Rosseting method)

EXPT.NO.9

DATE.

AIM:

To separate T & B lymphocytes from peripheral blood by E-rosette technique.

PRINCIPLE:

Human T cells have specific surface antigen CD-2 on this surface. This has a natural affinity for a sheep erythrocyte on incubating isolated lymphocytes with sheep blood. If will get attached to the receptor on T cell and under microscope it will look like floral structure called rosette to three or mare sheep RBC.

MATERIALS REQUIRED:

- Isolated sample lymphocytes
- Sheep blood collected in alsever's solution
- Centrifuged tubes
- Haemocytometers.

PROCEDURE:

1. Human and sheep blood was taken in 1:1 ratio and incubated for 10 minutes at 37° C.
2. A drop of suspension was taken under microscope for rosette.
3. Centrifuge the suspension at 1000 rpm for 10 minutes to sediment the rosette.
4. Collect the supernatant containing B-cells and counted in haemocytometer.
5. Lyse the sheep RBC's and count the 'T' cells in haemocytometer.

OBSERVATION:

Flower like rosette were seen on. Incubation of isolated lymphocytes with sheep RBC.

RESULT:

Human T-cell and B-cell lymphocytes were separated by E-rosette technique.

ELISA (SANDWICH)

EXPT.NO. 10

DATE.

AIM

To perform quantification of antigen using ELISA

MATERIALS REQUIRED

Titer plate, micropipette, glasswares, PBS, saline, primary antibody, antigen, conjugate , substrate, blocking buffer, washing buffer, substrate buffer, coating buffer, distilled water.

1. **Coating Buffer**

Solution A - 1.0 M $NaHCO_3$ 8.4 g / 100ml
Solution B - 1.0 M Na_2CO 10.6 g / 100ml.
Mix 45.3 ml Sol A
18.2 ml Sol B
Adjust the pH to 9.6

2. **10 X Phosphate Buffered Saline (PBS).**

KCl	2.0 g
NaCl	80.0 g
Na_2HPO_4. 7 H_2O	21.7 g
KH_2PO_4	20.0 g

Add distilled water upto 1 litre and adjust the pH to 7.4 with 10 N NaOH.

3. **Blocking solution**

10 X PBS pH 7.4	50 ml
0.05% Tween -20	0.25 ml
5% BSA	25 g.

Add distilled water up to 500 ml, filter

4. **ELISA Diluent**

10 X PBS pH 7.4	100.0 ml
0.05% Tween - 20	0.5 ml
1% BSA	10.0 g.

Add distilled water up to 1 liter, filter.

5. **Washing Buffer**

10 X PBS pH 7.4	400 ml
0.05% Tween-20	2 ml.

Add distilled water and make up to 4 liters.

6. *Substrate Diluent*

$NaHCO_3$	1.69 g
Na_2CO_3	2.51 g
$Mg\ Cl_2$	0.41 g.

Add distilled water and make up to 2 liter, pH 8.6.

PROCEDURE

ANTIBODY COATING

- The antibody was diluted in coating buffer provided at 1:100 dilutions and 100ul of antibody was added to each well of ELISA plate
- The plate was left at 4C overnight for passive absorption of antibody to the ELISA plate

WASHING

- Unabsorbed antibody need to be removed after overnight incubation
- Washing is done using ELISA washer or manually by adding 300ul of washing buffer to each well and discarding it.
- Manual washing is repeated thrice after which the plate is flipped to completely drain the washing buffer.

BLOCKING

- Blocking buffer was then added to the wells (300µl/well). Blocking buffer was added to prevent cross reactivity.
- Proteins in blocking buffer (BSA) bind to unbound sites in microtiter plate, so that these sites are not exposed during the course of the reaction process.
- The plate was incubated at 37°C for 1 hours.
- After 1 hour manual washing was repeated thrice.

ANTIGEN DILUTION

- The antigens that need to be quantified is serially diluted in PBS.
- Serially double dilution from 1:100 to 1:1600 was performed.
- Similarly the sample antigen was diluted in 1:100 in PBS.
- Diluted standard and sample antigen was added in duplicated. A set of well was left empty which is the blank.
- The plates were incubated at 37C for 1 hour.

CONJUGATE ADDITION

- After incubation, the plate is washed thrice manually and 100ul of diluted conjugate (detection antibody/ enzyme tagged) was added to all wells and incubated at 37C for 1 hour.
- Washing step was repeated following incubation

SUBSTRATE ADDITION

- 100ul of diluted substrate was added to all wells of ELISA plate and the plate was incubated at 37°C dark for 10 min's.
- The reaction was then stopped by addition of 25ul of stopping solution.

RESULT:

IMMUNOFLUORESCENCE PROCEDURE

EXPT.NO. 11

DATE.

PRINCIPLE

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. The specific region an antibody recognizes on an antigen is called an epitope. There have been efforts in epitope mapping since many antibodies can bind the same epitope and levels of binding between antibodies that recognize the same epitope can vary. Additionally, the binding of the fluorophore to the antibody itself cannot interfere with the immunological specificity of the antibody or the binding capacity of its antigen. Immunofluorescence is a widely used example of immunostaining (using antibodies to stain proteins) and is a specific example of immunohistochemistry (the use of the antibody-antigen relationship in tissues). This technique primarily makes use of fluorophores to visualise the location of the antibodies.

Samples:

1. Cultured cells: Cells may be grown on a 12mm round coverslips and stained in the wells of a 24-well plate. Alternatively cells can be grown in a petri dish, in which a hole has been made and a coverslip is glued in (Mat-Tek Corp. 200 Homer Ave, Ashland MA 01721, (800) 834-9018). Finally, now that we have an upright confocal microscope cells maybe grown on plastic and viewed live with the Zeiss LSM 510 Meta using dipping objecives.
2. Sections of fixed tissues: Sections up to 200 µm thick made with a Vibrotome can be viewed on a 2-photon microscope. For standard confocal (single photon), 50-100 µm should be max.
3. Thin sections: These should be made as usual for histological staining. After attaching to glass slide (coated with poly-L-lysine or purchased charged slides: Vector Labs), they must be fixed.

Fixation:

Since different fixatives can have various effects on cytoskeletal structures or on proteins by cross-linking, several methods of fixation should be tested for each antibody developed:

1. 3.7% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature followed by 0.2% Triton X100 in PBS (5 min) or 100 µM digitonin (5-10 min) permeabilization. This is the fix of choice if it works with your antibodies, because the cytoskeleton is best preserved. (Permeabilization may not be necessary in very thin sections.)
2. Absolute methanol at -20°C for 6 minutes; flood with PBS-0.5% BSA to prevent drying when incubation is finished.

3. Absolute ethanol at room temperature for 30 seconds; flood with PBS-0.5% BSA to prevent drying when incubation is finished.

Fluorescence staining:

1. Wash the cells several times in PBS-0.5% BSA. (I just use a squirt bottle and thoroughly flush the slide several times or fill and dump in the case of cells grown on coverslips and incubated in 24-well plates.)
2. Incubate with primary antibodies in PBS-0.5% BSA for 1 hour at room temperature or a half hour at 37°C. I usually only make enough working dilution for each experiment and don't reuse it or save left over. The total amount I use for 24-well plates or for circles drawn on slides is 10µl. But I don't measure less than a microliter of stock; so if it should be diluted 1:200, I make 200 µl. If there are two antibodies I mix them first, then place the solution on the cells. Whether on a slide or on coverslips in wells this incubation should be done in a humid chamber. This can be accomplished by placing the slide on toothpicks on a damp paper towel in a plastic box of some sort, or placing the damp paper towel in the lid of the 24-well plate.
3. After incubation, wash again in PBS, and add fluorescent secondary antibodies (at this point I add the direct labeling reagents, eg DAPI or phalloidin, as well), followed again by washing. Only secondary antibody should be incubated on one sample to control for nonspecific reaction. Incubate and wash again as above.

Mounting:

They are embedded in an anti-fade media (available from Molecular Probes or Vector labs). There are of course many variations on this media. It is only important that the issue of fading be addressed somehow. I will often rinse my cells for preparation once in deionized water before mounting too. This simply rinses off any salt residue, but is not necessary for viewing. Thick sections of 60-200 µm may be stored in a PBS a solution containing 2% DABCO instead of mounting.

RESULT: