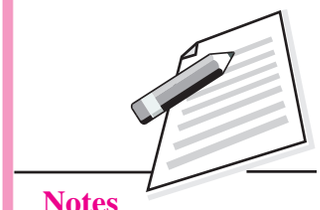


62

IMMUNOFLUORESCENCE

62.1 INTRODUCTION

Immunofluorescence (IF) is one of the very common laboratory techniques used in almost all disciplines of Biology including Medicine for diagnostics and research. Way back in 1942 Coons and Kaplan did some experiments and reported that the fluorescence dyes can be conjugated with antibodies and these labeled antibody can be used as probes to detect and locate the antigen specific to this antibody. Later this technique was named the Direct Fluorescent Antibody test. This technique has many applications and has been used in diagnostics and research. Immunofluorescence (IF) is used to detect specific proteins in cells that may be in suspension (specimen), in culture, in tissues, on microbeads and microarrays, etc. Fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) are the common fluorescent dyes which are chemically conjugated with the antibody. The FITC labeled antibody can be made to bind directly with the specific antigen (Direct fluorescence antibody test) or can be made to bind indirectly with an antigen (Indirect fluorescence antibody test).

Direct immunofluorescence is used less frequently as the antibody against the molecule of interest is chemically conjugated to a fluorescent dye, so for every antigen to be detected, the specific antibody will have to be conjugated with FITC. In indirect fluorescence the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye. Indirect fluorescence is used more commonly as the tagged secondary antibody and can be used to detect many different antigens. However, the primary antibody will have to be specific for the antigen to be detected.

The fluorescence can be read as a qualitative result or quantitative result using fluorescence microscopy. The fluorescence can also be quantified using a flow cytometer, array scanner or automated imaging instrument.

Notes

Recently a couple of advances have been made in fluorescence technology. One is an improvement in optical filters, and the second is the use of fiber optics for measuring fluorescence emission from small organisms. The optimum wavelength for exciting fluorescence in FITC is 495 nanometers. FITC absorbs most of light at 495 nanometers and then emits light at 525 nanometers. Till recently and even now mostly ultraviolet or near ultraviolet light source is used to excite fluorescence as the wavelength usually selected (365 nanometers) can be separated easily from the emitted light at 525 nanometers.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the history of immunofluorescence
- discuss the principle of immunofluorescence
- describe the various types of immunofluorescence
- describe the methods of various types of immunofluorescence with examples
- practice "Quality control" for immunofluorescence
- interpret the result of immunofluorescence
- describe the various applications of immunofluorescence.

62.2 DEFINITION

Immuno Fluorence is defined as various techniques used for detecting an antigen or antibody in a sample by coupling its specifically interactive antibody or antigen to a fluorescent dye/compound, mixing with the sample, and then observing the reaction under an ultraviolet-light fluorescence microscope.

The antibodies and or antigens are chemically linked to a fluorescence dye to identify or quantify interactive antigens and or antibodies in a cell/tissue sample/ culture, etc.

A fluorescence microscope (Fig. 62.1 and Fig. 62.2) is required to read the result of the test. There are a number of fluorochromes (fluorescence dyes) which can be used in fluorescent microscopy. Different colours are emitted by different dyes. FITC emits bright, apple green fluorescence as shown in Fig. 62.3.

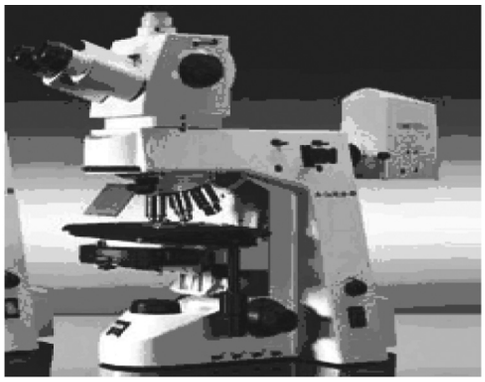


Fig. 62.1: Fluorescence microscope

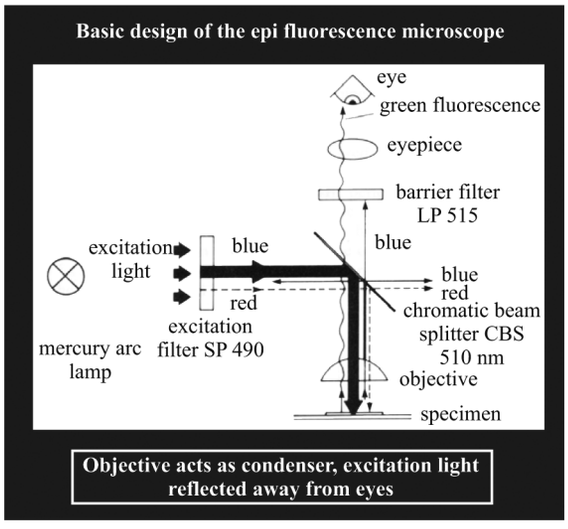


Fig. 62.2: Basic structure of a fluorescence Microscope

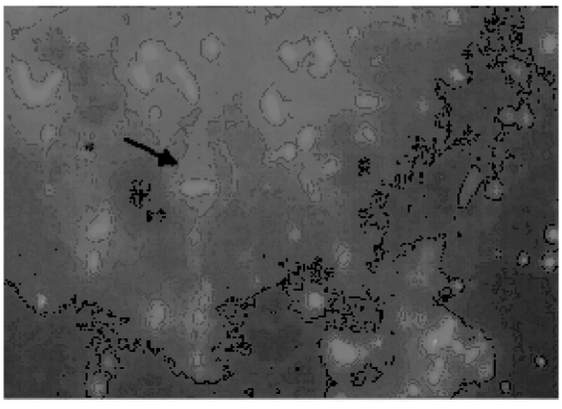


Fig. 62.3: Immunofluorescence- Positive result is bright, apple green fluorescence in case of FITC.

62.3 PRINCIPLE OF IMMUNOFLUORESCENCE

We will first understand the basics of fluorescence and then go on to understand immunofluorescence.

62.3.1 Fluorescence

Fluorescence is a type of luminescence. The fluorescent dyes/fluorochromes (having luminescent properties) absorb light of one wavelength and emit light of a different wavelength extremely rapidly. Absorbed light has a higher energy than the emitted fluorescence light, so the wavelength of the emitted light is longer than that of the excitation light. Blue light (between 450 nm and 520 nm, 495 nm is optimal) is the excitation spectrum and green light is the emission spectrum (490 nm to 630 nm, and the emission peak is approximately 515 nm). An atom has electrons each of which has predetermined level of energy. An electron can absorb energy from a photon of light and become excited. The energy level is higher in the excited stage, but this stage is unstable. The excited electron emits fluorescence; the energy of electron at this stage is lower than when in excited stage. This produces the magnified fluorescent image of the object which can be visualized with fluorescence microscope.

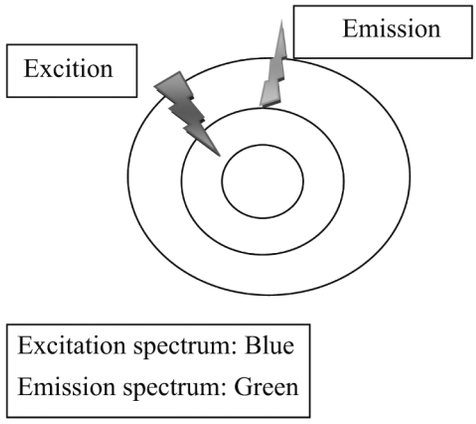


Fig. 62.4

INTEXT QUESTION 62.1

1. ..... are techniques used for detecting antigen/antibody by coupling them with fluorescent dye
2. ..... absorb light of one wave length & emit light of a different wavelength

3. ..... is the excitation spectrum

4. ..... is the emission spectrum

62.3.2 Immunofluorescence (IF)

In immunofluorescence usually the fluorochrome like FITC is tagged to antibody which in the test (the fluorescence antibody technique) will bind to corresponding specific antigen/DNA/chemical present in the sample (bacteria, virus, parasite, tissue, cells, etc.). The reaction is then read on fluorescence microscope using blue light and appropriate filters to see the bright green fluorescence in case of a positive result.

Some of the fluorochromes can enter the living cells and others cannot. The former type are used to differentiate the living and dead cells.

IF is of two types direct and indirect. Indirect IF is used most commonly. In indirect fluorescence the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye. Immunofluorescence uses antibodies as fluorescent probes to visualize antigens within cells. The technique is highly sensitive as well as specific.

62.4 FLUORESCENCE MICROSCOPY

For undertaking Fluorescence microscopy take into consideration the light sources, the filters and the objectives of the Fluorescence Microscope to be used.

The light microscope used routinely in Microbiology uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample. However, as mentioned earlier also fluorescence microscope, uses a high intensity light source for excitation of the fluorochrome tagged sample of interest and emits a lower energy light of a longer wavelength that produces the magnified fluorescent image.

Fluorescence microscopy is used in almost all fields of biology and medicine for diagnostics and research.

62.4.1 Auto fluorescence

Certain biological structures such as mitochondria, riboflavin, melanin, elastin and collagen, etc. fluoresce after absorbing light, emit light with fluorescence without the addition of fluorophores like FITC. This is due to auto fluorescence i.e. the structures are fluorescing by themselves.

Auto fluorescence interferes with the reading of test result in cases where the specimen contains structures that auto fluoresce by causing unwanted background weak signals.

62.4.2 Photobleaching

Exposure of fluorescence stained sample to light causes photochemical destruction of the fluorescence dye (FITC or some other fluorophore) and this phenomenon is called photobleaching. In diagnostics photobleaching can result in erroneous/ wrong result. We should be careful that after fluorescence staining the result is read immediately or the slide is kept protected from light/in dark in refrigerator. It is important for you to understand that the fluorescence microscopy test result must be read on the same day.

62.5 MATERIALS REQUIRED FOR CARRYING OUT IMMUNOFLUORESCENCE TESTS

Now you know that immunofluorescence has many applications in diagnostics and research. It is not possible to discuss all the various applications of immunofluorescence. We will take two examples one of detection of auto antibodies against ds DNA and another of detecting Pneumocystis jirovecii in broncheo alveolar Lavage.

Whenever you undertake direct or indirect IF staining follow the instructions given in the kit insert and perform read and interpret the result accordingly.

62.5.1 Materials required for detection of auto antibody against ds DNA in serum

Sample type: Blood Serum

Equipment and plastic wares:

- Fluorescence microscope with the light source
- Magnetic stirrer and magnetic beads
- Centrifuge
- Distilled or de-ionized water
- Pasteur pipettes = 0.5-10ul, 10-100ul, 100-1000ul
- 500 ml measuring cylinder 10. 500 ml conical flask

Reagents:

- **Substrate slides**: Crithidia luciliae

- **Positive control**: Pooled human serum with a specific autoantibody activity with 1% bovine serum albumin with 0.1% sodium azide
- **Negative Control**: Pooled normal human serum with 1% bovine serum albumin and 0.1% sodium azide
- **FITC Conjugate**: Fluorescein conjugated antiserum to human immunoglobulins with 1% bovine serum albumin, 0.1% sodium azide;
- Mounting media
- Phosphate buffered saline (PBS): pH 7.3± 0.10
- Blotting strips.

62.5.2 Materials required for detection of Pneumocystis jirovecii in broncheoalveolar lavage:

Sample type Broncheo Alveolar Lavage (BAL)

The sensitivity of BAL specimen for detection of *P. jiroveci* is 90%-99%. BAL should be performed in two areas of lungs including the upper lobes. After the bronchoscope is placed and wedged, normal saline is instilled in 50 ml aliquots and immediately withdrawn with suction or syringe. The recovered specimen is centrifuged and stained for *P. jiroveci*.

Equipment and other materials

- Fluorescence microscope with filter system for FITC and Evans blue
- Glass coverslips (24X60mm)
- Automatic pipettes
- Petridish with moistened filter paper for moist incubation
- Staining cuvettes
- Vortex mixer
- Screw capped centrifuge tubes

Reagents

- Commercially available kit with following reagents:
  - (a) FITC conjugated mouse anti-Pneumocystis jirovecii monoclonal antibodies
  - (b) Buffered glycerine mounting medium
  - (c) Phosphate buffered saline (PBS)

The basic requirement is the fluorescence microscope for carrying out IF tests, the other equipment and materials required will be as per the kit insert for the test being undertaken.

Notes

62.6 METHOD OF IF STAINING

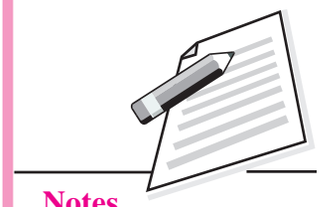
The method of the IF test will be as per the details given in the kit insert of the test being performed. We are giving below as examples, the methods for the same two tests (detection of autoantibody against ds DNA and detection of P jirovecii) for which the requirements have been detailed above.

62.6.1 Procedure for ds DNA auto antibody detection

- Remove appropriate number of slides from freezer. Equilibrate to room temperature in foil bags (20 minutes). Bring all reagents to room temperature
- Reconstitute PBS with distilled water in clean and contamination free container
- Prepare screening dilutions (1:10) of the test sera in PBS. Mix by inversion
- Remove the reagent slide from foil bag and place in Petri dish. Immediately add 25-35 µl controls or diluted test sera to wells. Repeat this step for all the slides
- Incubate substrate slides at room temperature for 20 minutes
- Rinse each slide briefly with a stream of PBS
- Wash slides for a total of 10 minutes in Petri dish filled with PBS
- Remove each slide from Petri dish and blot excess PBS from around wells
- Place slide in new Petri dish and immediately dispense approximately 25µl FITC Conjugate to each well. Repeat this step for all the slides
- Incubate 20 minutes at room temperature in dark
- Rinse each slide briefly with a stream of PBS
- Wash slides for a total of 10 minutes in Petri dish filled with PBS. Remove each slide from PBS and drain briefly on a paper towel. Apply drops of mounting media per slide, making sure to cover all wells and mount the slides, cover with coverslip
- Analyze the slides under a fluorescent microscope in a dark room. Evaluate each substrate well for the presence or absence of auto antibodies
- Note the result

62.6.2 Detection of P jirovecii in BAL:

- Centrifuge 5 ml of the Lavage or bronchial washings in screw capped tubes at 3000 xg for 10 min. Discard supernatant. Resuspend the sediment in 0.5 to 1.0 ml of sterile 0.05 M Tris-HC1 buffer (PH 7.5) and vortex for 30 seconds.



- Smears are made from the sediment
- Air dry the smear
- Fix smear by covering with cold acetone (-20°C) for 5-10 min
- Bring all the kit reagents to room temperature
- Cover the smear prepared from the specimen as above with 30µl of anti-Pneumocystis MAB(reagent)
- Place the slide in a humid chamber (pertridish containing moist cotton) and incubate for 30 min at room temperature
- Immerse the slide in PBS for 5 min
- Air dry the smear
- Add 3 drops of mounting fluid over the dried smear and cover with a cover slip
- Examine the slide with fluorescence microscope under 40x or 100x objectives

62.7 LABORATORY INTERPRETATIONS OF RESULTS

The slides are read using a fluorescence microscope in a dark room

62.7.1 ds DNA auto antibody detection

1. Read only single, well defined organisms in each field. Not all organisms will appear optimal due to orientation on the slide, individual stages of growth and microscope viewing hindrance
2. The kinetoplast is oriented toward the flagellum of the Crithidia luciliae. A positive staining kinetoplast may appear as a solid fluorescent disk or doughnut-shaped with more intense staining at the edges than in the middle. The nucleus is larger and can fluoresce but it is not specific for ds DNA auto antibodies. The nucleus may or may not show positive fluorescence but do not use nuclear fluorescence as a criterion for the determination of auto antibody
3. Five types of staining may result:
   - No fluorescent staining of kinetoplast or nucleus. Interpretation: negative.
   - No fluorescent staining of kinetoplast but positive fluorescence of nucleus. Interpretation: negative for auto antibody.
   - Fluorescent staining at the base of flagellum of organism. Interpretation: negative. This is non-specific reaction.

- Flourescent staining of kinetoplast only. Interpretation: positive for auto antibody. Report a positive reaction for a particular sample dilution only if the kinetoplasts of the majority of organisms fluoresce.
- Fluorescent staining of kinetoplast and nucleus. Interpretation: positive for auto antibody. Report a positive reaction at a particular sample dilution only if the kinetoplasts of the majority of organisms fluoresce.

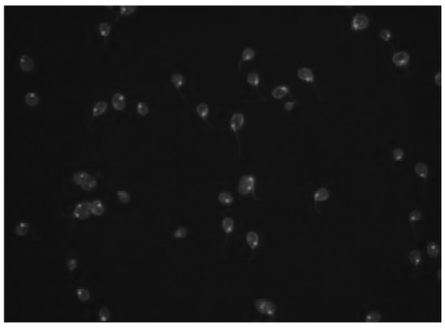


Fig. 62.5: ds DNA Ab positive-Kinetoplast of Crithidia luciliae showing fluorescence

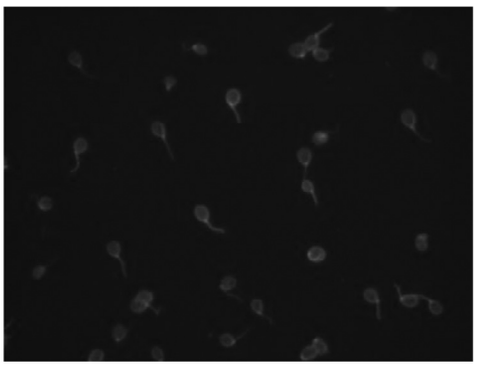
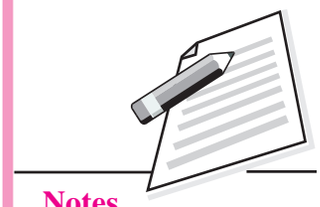


Fig. 62.6: ds DNA Negative-Kinetoplast of *Crithidia luciliae* not showing any fluorescence

62.7.2 Detection of P jirovecii

Interpretation

The Pneumocystis organism are visible either as single or aggregates of extra cellular thick walled cysts with bright apple green fluorescence in the foamy alveolar exudate. The cysts aggregate may or may not be embedded in a brightly stained extra cellular matrix. Individual cysts may show a comma shaped or parentheses like structure. Cysts usually show peripheral green fluorescence. Other developmental stages like mature sporozoites/trophozoites and precysts may also be seen.



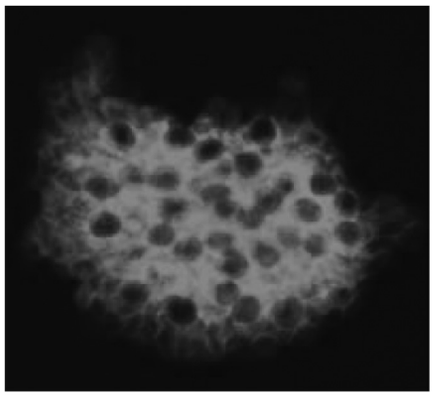


Fig. 62.7: P jirovecii IF positive

62.8 QUALITY CONTROL

The quality control procedure for almost all IF tests are the same i. e. negative and positive kit controls are used to assure the quality of performance of test. The results of the negative and positive controls should always be correct, only then the test results are taken as valid.

62.8.1 Quality control ds DNA auto antibody detection:

- Positive and negative controls must be included in each run to confirm reproducibility, sensitivity and specificity of the test procedure
- The negative control serum should demonstrate no fluorescence of the kinetoplast
- The positive control serum should demonstrate 3+ to 4+ fluorescence of the kinetoplast. The kinoplast is oriented toward the flagellum of the Crithidia luciliae. A positive staining kinoplast may appear as a solid flourescence disk or doughnut-shaped with more intense staining at the edges than in the middle .The nucleus is larger and can fluoresce but it is not specific for anti-nDNA antibodies
- The positive and negative controls must demonstrate appropriate reactions otherwise the run is considered invalid and must be repeated

62.8.2 Quality control detection of P jirovecii:

A known positive slide is stained along with the test slide every time the staining is done and it should always give a positive result..

62.8.3 Safety precautions:

- All specimen used in this test should be considered potentially infectious. Universal precautions (gloves) should be used for handling and disposal of materials during and after use

- Use soap for routine hand washing
- Use biohazard waste disposal guidelines

62.9 APPLICATIONS

As has already been explained to you that out of the two IF techniques direct and indirect, the indirect IF is most commonly used. IF is used in biology and medicine. Medical applications include Diagnostics and research. We have given examples of detection of auto antibody to ds DNA (to diagnose auto immune disorder) and detection of P jirovecii in BAL (to detect the infection with Pneumocystis). You will find many applications of the IF technique in medicine, biology and research.

Basically application of IF is to visualize antigens within cells using specific antibodies as fluorescent probes and vice-versa. Various applications include:

- Resolution of details to the molecular level
- Study a cell population for viability (some fluorophores penetrate live cells and not the dead cells as already explained under microscopy)
- Detect specific cells of interest in a specimen/material using FISH techniques
- Viewing structural components of cells, bacteria, parasites, fungi and bacteria
- Imaging the genetic material within a cell (DNA and RNA)
- Defining the spatial-temporal patterns of gene expression within cells

INTEXT QUESTIONS 62.2

1. Auto fluorescent structures are ..... , ... & .....
2. Exposure of fluorescence stained sample to light couasing photochemical destruction is known as .....

WHAT YOU HAVE LEARNT

- Immunofluorescence is one of the very common laboratory techniques used in almost all disciplines of Biology including Medicine. Way back in 1942 Coons and Kaplan did some experiments and reported that the fluorescence dyes can be conjugated with antibodies and these labeled antibody can be used as probes to detect and locate the antigen specific to this antibody.

- Direct immunofluorescence is used less frequently as the antibody against the molecule of interest is chemically conjugated to a fluorescent dye, so for every antigen to be detected, the specific antibody will have to be conjugated with FITC. In indirect fluorescence the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye. Indirect fluorescence is used more commonly as the tagged secondary antibody can be used to detect many different antigens. However, the primary antibody will have to be specific for the antigen to be detected.
- IF is defined as various techniques used for detecting an antigen or antibody in a sample by coupling its specifically interactive antibody or antigen to a fluorescent dye/compound, mixing with the sample, and then observing the reaction under an ultraviolet-light fluorescence microscope.
- In immunofluorescence usually the fluorochrome like FITC is tagged to antibody which in the test (the fluorescence antibody technique) will bind to corresponding specific antigen/DNA/chemical present in the sample (bacteria, virus, parasite, tissue, cells, etc.). The reaction is then read on fluorescence microscope using blue light and appropriate filters to see the bright green fluorescence in case of a positive result.
- Some of the fluorochromes can enter the living cells and others cannot. The former type are used to differentiate the living and dead cells.
- Auto fluorescence interferes with the reading of test result in cases where the specimen contains structures that auto fluoresce by causing unwanted background weak signals.
- The method of the IF test will be as per the details given in the kit insert of the test being performed. We have given examples of tests (detection of autoantibody against ds DNA and detection of P jirovecii) for which the requirements, method, interpretation, quality control have been detailed above. Please go through the details to understand IF test.
- Basically application of IF is to visualize antigens within cells using specific antibodies as fluorescent probes and vice-versa. Various applications include:
    - Resolution of details to the molecular level
    - Study a cell population for viability (some fluorophores penetrate live cells and not the dead cells as already explained under microscopy)
    - Detect specific cells of interest in a specimen/material using FISH techniques

- Viewing structural components of cells, bacteria, parasites, fungi and bacteria
- Defining the spatial-temporal patterns of gene expression within cells
- Imaging the genetic material within a cell (DNA and RNA)

TERMINAL QUESTIONS

1. What is immunofluorescence? Describe the different types of immunofluorescence techniques.
2. Define IF and the basic requirements for IF.
3. Describe the principle of fluorescence and IF.
4. Define autofluorescence and photobleaching and enumerate the problems these cause in IF test
5. Describe the materials required to perform IF test for detection of auto antibody to ds DNA.
6. Describe how will you read and interpret an IF test.
7. Describe the various applications of IF.

ANSWERS TO INTEXT QUESTIONS

62.1

1. Immunofluroescence
2. Fluorescent dyes
3. Blue light
4. Green light

62.2

1. Mitochondria, riboflavin & collagen
2. Photobleaching