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Fluorescence Microscope: A Review Article

Temesgen Bihonegn
Department of Animal Health, Kombolcha Agricultural College, P.O. Box 56, Kombolcha, Ethiopia

SUMMARY
A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence to study properties of organic or inorganic substances. Therefore, the objective of this seminar paper is to understand the principles of fluorescence microscope and acquire knowledge about fluorescence microscope. The basic task of the fluorescence microscope is to let excitation light radiate the specimen and then sort out the much weaker emitted light to make up the image. Fluorescence microscopy is a rapid expanding technique, both in the medical and biological sciences. Therefore, laboratory practitioners and other basic science professions should know in details the principles and application of fluorescence microscope. The environment of the fluorochrome stained specimen from fading should be protected.
Keywords: Fluorescence Microscope, Fluorochrome, Image and Specimen

1. INTRODUCTION

A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances (Spring and Davidson, 2008). The fluorescence microscope refers to any microscope that uses fluorescence to generate an image, whether it is a simpler set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image (Spring and Davidson, 2008).

Fluorescence is light produced by a substance when it is stimulated by another light. Fluorescence is a short-term event; the fluorescent substance produces light only while it is being stimulated. Fluorescence microscopy is a way to discover facts about specimens that often are not visible to bright-field microscopy. In fluorescence microscopy, specimens are self-illuminated by internal light, so fluorescence shows bright objects in vivid colour against a dark background. Bright objects against dark backgrounds are more easily seen. Since the light comes from within the specimen, internal structures and certain substances can be seen and located. These characteristics make fluorescence microscopy a very sensitive and specific tool (Sanborn *et al*., 2005).

Figure 1. An upright fluorescence microscope (Olympus BX61) (Spring and Davidson, 2008).

Therefore, the objective of this seminar paper is to understand the principles of fluorescence microscope and to acquire knowledge about fluorescence microscope.

2. FLUORESCENCE MICROSCOPE

2.1. History of fluorescence microscope

The history of fluorescence microscopy started in 1904 when Köhler examined biological materials under a microscope using ultraviolet light. In 1938, Haitinger summarized the actual knowledge about the application of fluorochromes in microscopy. The birthday of immunofluorescence microscopy (fluorescent antibody) was in 1941, when Coons, Creech and Jones conjugated the fluorescent compound ß-anthryl isocyanate to pneumococcal antiserum.

Brumberg was the first to describe the epi-illumination microscopy technique in 1959. In 1967, Ploem reported a vertical illuminator and a dichroic mirror system that permitted passage of selected-wavelength light

through a dichroic mirror and reflected other wavelengths. In this technique the low-wavelength excitation beam is reflected downward and focused on the specimen through the objective lens, and the higher-wavelength emitted light is transmitted back through the objective to the eye through the dichroic mirror.

In 1967, Tomlinson reported a filter and lamp system to view fluorescent antibody preparations with a 100 watt quartz–iodine lamp. In 1968, Johnson and Dollhopf expanded on Tomlinson's work. Interestingly, the filter combination theyused gave satisfactory bright orange fluorescence from rhodamine conjugates.

Nairn (1968) summarized the development of fluorescence microscopy in the fourth edition of his publication Fluorescent protein tracing. Since then, development of more sophisticated techniques, such as laser fluorescence microscopy and confocal fluorescence microscopy, shows that fluorescence microscopy technology has not reached its end.

2.2. Advantage of Fluorescence Microscope

The advantages of fluorescence microscopy are due to its sensitivity, specificity, adaptability and easy use. Reliability is high because it is simple and can be well controlled. This makes fluorescence microscopy a very useful tool for teaching biology. The brilliant colours of fluorescence seen in illuminated specimens are very memorable for students (Sanborn *et al*., 2005).

The equipment needed for fluorescence microscopy ranges from very complex microscopes to relatively simple devices, most bright-field microscopes can be easily converted into fluorescence microscopes. Using fluorescence microscopy can reduce laboratory testing costs. Fluorochrome dyes are effective at very dilute concentrations, 1:10 000 or greater. Thus, the cost per test is low (Sanborn *et al*., 2005).

Fluorescence microscopy offers a number of advantages, especially for medical diagnostic tests. These are as follows.

Easy: Preparing and staining fluorescence test specimens is as easy as, or easier than, conventional slide staining. Examining fluorescent specimens is easy because the contrast is excellent (Sanborn *et al*., 2005).

Sensitive: Due to the relatively bright fluorescent image against a dark background, small objects are easy to see (Sanborn *et al*., 2005).

Specific: By using modern fluorochromes and interference light filters, high specificity for details is possible (Sanborn *et al*., 2005).

Rapid: Specimens can be examined directly on a slide, often without any prior purification or concentration (Sanborn *et al*., 2005).

Reliable: Good reliability and accuracy of microscopic analysis are due to the high sensitivity and specificity of fluorescence microscopy techniques (Sanborn *et al*., 2005).

*Universa*l: Fluorescence microscopy can be applied to a wide range of biomedical disciplines such as bacteriology, mycology, virology, parasitology, serology, immunology, autoimmunology, cytology, cell biology and histochemistry (Sanborn *et al*., 2005).

Adaptable: Fluorescence microscopy methods can replace and improve many conventional bright-field microscopy tests. Most compound microscopes can be used for fluorescence microscopy, provided the instruments are equipped with a few fluorescence accessories (Sanborn *et al*., 2005).

2.3. Applications of Fluorescence Microscopy

It permits users to easily see very small cells and their contents in microscopic specimens with outstanding detail. Fluorescence microscopy often reveals features in a specimen that cannot be seen by standard bright-light microscopy, especially internal structures. As a result of the exceptionally low fluorochrome concentrations needed for secondary fluorescence (1:10 000 or higher), fluorochromes are safely used to examine living tissue and microorganisms. The applications of fluorescence microscope is summarized below (Table 1) (Sanborn *et al*., 2005).

Table 1. Fluorescence microscopy applications

Infectious disease diagnosis Agent detection and identification Antibody titration Toxin detection	*Industrial process control* Monitoring of clean rooms Detection of fluid contamination Sampling of food and beverages
Autoimmune disease diagnosis Antibody detection and identification Antibody titration	*Disease diagnosis* Etiological agent detection and identification Antibody titration
Environment monitoring Microbial aerosol monitoring Water analysis Limnology	*Environment microorganism monitoring* Air testing (aerosols) Water analysis (bacteriological safety) Sewage treatment control
Emergency support Casualty diagnosis Microorganism aerosol detection and identification	*Biological attack defence* Rapid diagnosis of casualties Detection of biological aerosols Sampling of suspicious munitions
Biological research Location and identification of cells Identification of tissues Detection of nucleic acids	*Industrial process control* Monitoring of clean rooms (electronics) Detection of fluid contamination (electronics, pharmaceuticals) Detection of product contamination (food, beverages)

Source: (Sanborn *et al*., 2005).

2.4. Basic Fluorescence Microscope Parts

The equipment needed for fluorescence microscopy comprises:

A compound microscope is the platform that brings together all the other parts and provides magnification and resolution of the specimen image. A light source supplies bright light of a suitable wavelength (colour) to stimulate fluorescence.An excitation (primary)filtertransmits only the light wavelength (colour) needed to excite fluorescence of the fluorochrome used. It blocks other wavelengths from the light source that are not needed. A dichroic mirror further reflects the exciting light colour, but it passes the higher wavelength fluorescence light. A barrier (secondary) filter blocks any exciting light that may escape the other filters, and it also passes the higher wavelength fluorescence light(Sanborn *et al*., 2005).

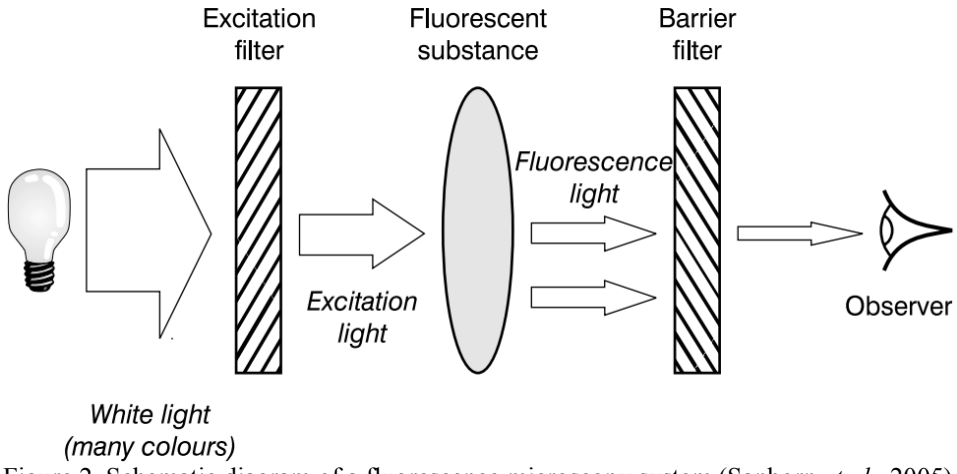

Figure 2. Schematic diagram of a fluorescence microscopy system (Sanborn *et al*., 2005).

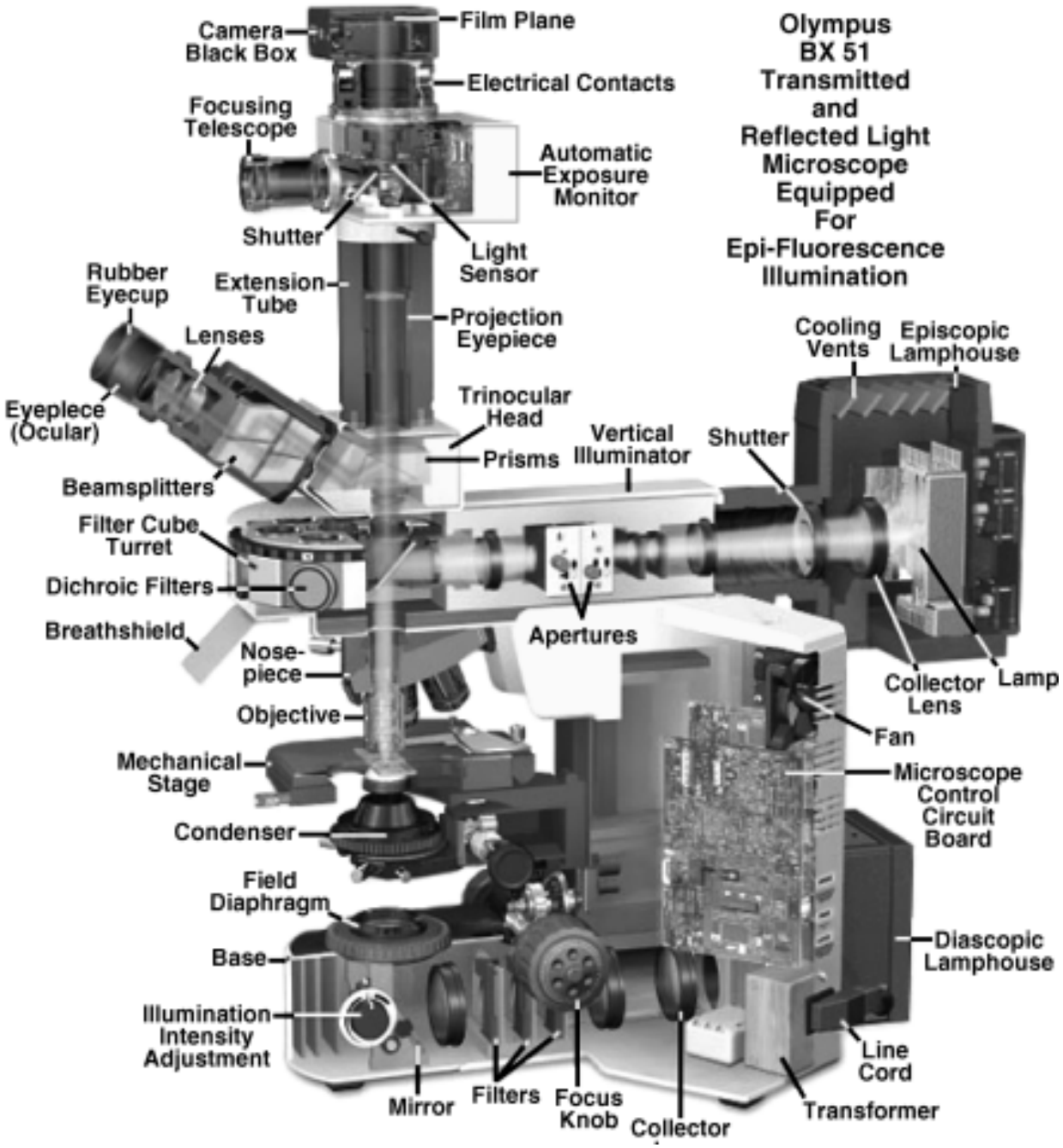

Fig. 3. Cut-away diagram of an upright microscope equipped both for transmitted light and epi-fluorescence microscopy. The vertical illuminator in the center of the diagram has the light source at one end (episcopic lamphouse) and the filter cube at the other(Spring, 2003).

2.5. Principle of Fluorescence Microscope

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images (Spring and Davidson, 2008).

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These

microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF) (Spring and Davidson, 2008).

Light is electromagnetic energy that moves through space as massless particles called photons. The distance between a point on one wave, for example the peak, and the same peak on the next wave is the called the wavelength. The energy is inversely proportional to the wavelength-the shorter the wavelength, the more energy. Wavelengths of the visible light spectrum range from about 400 nm to 750 nm. Different light wavelengths of visible light are seen by humans as different colours. Longer wavelengths of light above 750 nm are invisible light called infrared Shorter wavelengths of light below 400 nm are a higher energy invisible light called ultraviolet. Near ultraviolet means wavelengths just shorter than 400 nm, and far ultraviolet means even shorter wavelengths, about 350 nm and less. Infrared light has relatively low energy and low penetrating power, while the shorter-wavelength ultraviolet light has greater energy and penetrating power. The energy spectrum continues, and radiation with wavelengths shorter than ultraviolet light, such as gamma rays and X-rays, have still more energy and deeper penetrating power (Sanborn *et al*., 2005).

When light radiation of high enough energy strikes a substance that can fluoresce, the substance absorbs that energy and converts a small part of it into vibrational energy (i.e. heat). The energy that is not absorbed by the substance is emitted again as light. The emitted light is called fluorescent light. The wavelength of the emitted light is always longer than the wavelength of the generating light. This phenomenon is called Stokes shift. Fluorescence is short duration, and fluorescent substances emit light without noticeable delay and only while being stimulated (Sanborn *et al*., 2005).

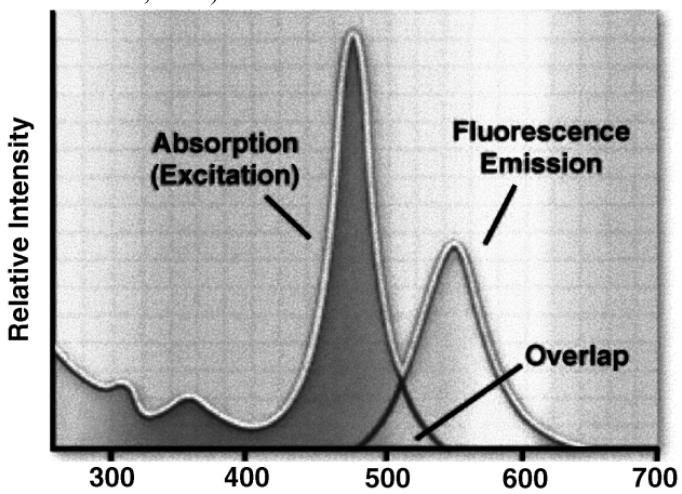

Fig. 4. Absorption and emission spectra are shown for fluorescein (Spring, 2003).

2.6. Primary and Secondary Fluorescence

A fluorescent substance can be excited by invisible ultraviolet light, and it will emit longer-wavelength visible light, for example, violet, blue, green or red light. However, many fluorescent substances are also excited by visible violet, blue or green light. The commonly used dye fluorescein is a good example. Exciting this fluorescent dye with blue light yields yellow-green fluorescence light (Sanborn *et al*., 2005).

Substances that can be activated to fluoresce are called fluorochromes. Fluorochromes may be naturally present in biological materials or may be artificially introduced into these materials. Fluorescence emission from untreated materials is known as primary, natural, self- or autofluorescence. When fluorescent substances are artificially introduced into a specimen, the emitted light is called secondary fluorescence (Sanborn *et al*., 2005).

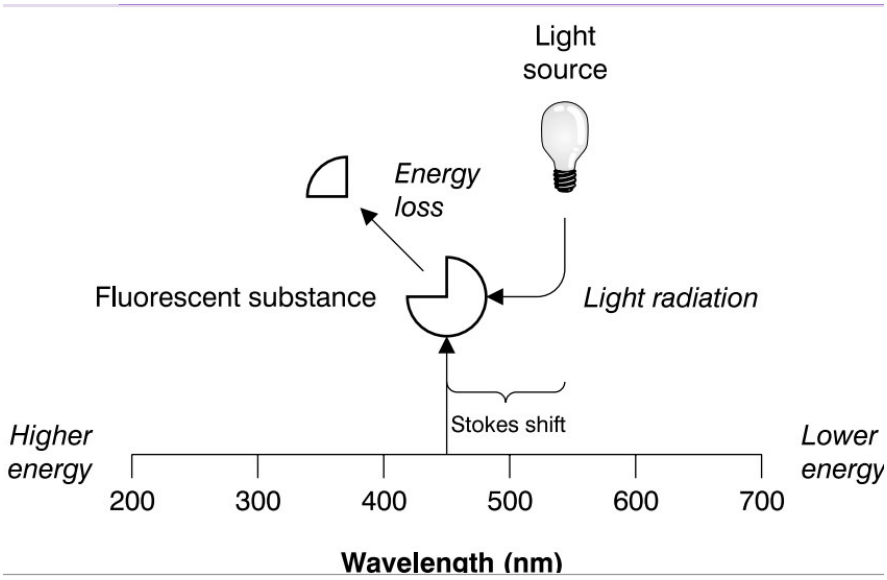

Figure 5. Scheme showing the origin of fluorescence radiation (Sanborn *et al*., 2005).

2.7. Light Sources for Fluorescence Microscope

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide field epifluorescence microscopes (Spring and Davidson, 2008).

2.8. Light Filters for Fluorescence Microscopy

Light filters are essential in fluorescence microscopy for selecting appropriate colour wavelengths to stimulate fluorescence and to block unwanted excitation light from the observer's eye. Filters provide wavelength colour selection. They can be changed for different microscopy applications. Various types of light filters are used. Light filters fall into several types; short band pass, long band pass, wide band pass, narrow band pass and dichroic(Table2) (Sanborn *et al*., 2005).

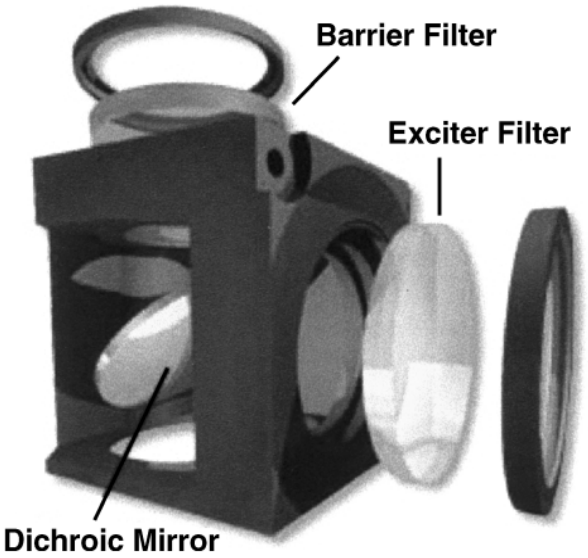

Fig. 6. A microscope filter cube containing the exciter and barrier filters as well as the dichromatic mirror (Spring, 2003).

Table 2. Light filters and their uses in fluorescence microscopy

Filter type	Use	Filter method
Short band pass	Excitation	Colour absorption or interference
Long band pass	Barrier (blocking)	Colour absorption or interference
Wide band pass	Excitation	Colour absorption or interference
Narrow band pass	Excitation	Barrier (blocking), interference
Dichroic	Beam-splitting mirror	Colour beam-splitting (epi-illumination)

Sources: (Sanborn *et al*., 2005).

2.9. Properties of Fluorochromes

A fluorochrome is a chemical dye that fluoresces (emits light) when it is stimulated (excited) by certain wavelengths (colours) of light. There are many hundreds of fluorochromes, and new ones are being synthesized to meet new requirements. Some are general fluorescent stains, staining a wide variety of tissues and substances. Others specifically stain certain cells, tissues or structures based on factors such as specimen pH, viscosity or the presence of certain chemicals. Others differentiate between living and dead cells. Some are specific for white blood cells, mitochondria or chromosomes. Certain fluorochromes can be attached (conjugated) to antibodies to form immunologically specific stains. This combination is called a fluorescent antibody, and the technique is known as immunofluorescence (Sanborn *et al*., 2005).

Table 3. Commonly used fluorochromes, their characteristics and applications.

Fluorochrome	Absorption peaks (nm)	Emission peaks (nm)	Staining applications
Acridine orange	460, 500	525, 650	DNA, RNA, cells, general bacteria, acid-fast bacteria, fungi
Auramine O	460	550	Acid-fast bacteria
Berberine sulfate	430	550	General bacteria
Calcophor white	365 (UV)	435	Fungi in tissue
Coriphosphine O	460	575	Leukocytes, bacteria, mucus
Dimethylaminonaphthalene-5-sulfonic acid (DANS)	340 (UV)	525	Antigen–antibody reactions
Evans blue	550	610	Fluorescent counterstain (red)
Fluorescein isothiocyanate (FITC)	490	525	Antigen–antibody reactions
Lissamine-rhodamine B (RB 200)	570	590	Antigen–antibody (RB-200) reactions, fluorescent counterstain (red)
Texas Red	595	615	Antigen–antibody reactions
Tetramethylrhodamine isothiocyanate (TRITC)	555	620	Antigen–antibody reactions

Source:(Sanborn *et al*., 2005).

2.10. Sample Preparation

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e., autofluorescence) can be used(Spring and Davidson, 2008). In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples (Spring and Davidson, 2008).

2.11. Biological Fluorescent Stains

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst which bind the minor groove of DNA, thus labelling the nuclei of

cells. Others are drugs or toxins which bind specific cellular structures and have been derivatised with a fluorescent reporter. A major example of this class of fluorescent stain is phalloidin which is used to stain actin fibres in mammalian cells (Spring and Davidson, 2008).

2.12. Immunofluorescence

Immunofluorescence is a technique which uses the highly specific binding of an antibody to its antigen in order to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody specific for the molecule of interest. A fluorophore can be directly conjugated to the primary antibody. Alternatively a secondary antibody, conjugated to a fluorophore, which binds specifically to the first antibody can be used (Spring and Davidson, 2008).

2.13. Fluorescent Proteins

The modern understanding of genetics and the techniques available for modifying DNA allows scientists to genetically modify proteins to also carry a fluorescent protein reporter. In biological samples this allows a scientist to directly make a protein of interest fluorescent. The protein location can then be directly tracked, including in live cells (Spring and Davidson, 2008).

2.14. Preventing Fluorescence Fading (Photobleaching)

When a fluorochrome is excited by light to fluoresce, the fluorescence is bright at first and then begins to fade with continued excitation. This is because a molecule of fluorochrome can emit only a certain number of photons during its photochemical lifetime. Fluorescence fading is caused by the interaction of light and oxygen on the stained specimen. Oxygen bleaches the fluorochrome faster or more slowly, depending on the light intensity. Low light level excitation does not prevent fading; it simply reduces the rate of fading (Sanborn *et al*., 2005).

Protection from fading (slowing the process) can be done three ways: reduce exposure time to the excitation light; reduce intensity (brightness) of the excitation light and deoxygenate the environment of the fluorochrome-stained specimen (Sanborn *et al*., 2005).

3. CONCLUSION

The basic task of the fluorescence microscope is to let excitation light radiate the specimen and then sort out the much weaker emitted light to make up the image. Fluorescence microscopy is a rapid expanding technique, both in the medical and biological sciences. The technique has made it possible to identify cells and cellular components with a high degree of specificity. The advantages of fluorescence microscopy are due to its sensitivity, specificity, adaptability and easy use. Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path.

Hence, based on the above conclusion the following recommendations are forwarded.

- Laboratory practitioners and other basic science professions should know in details the principles, techniques and application of fluorescence microscope.
- The environment of the fluorochrome-stained specimen from fading should be protected.

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