**Lec(3) Immunotechnolgy**

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**FLUORESCENT IMMUNOASSAY**

In 1941, Albert Coons demonstrated that antibodies couldbe labeled with molecules that fluoresce. These fluorescent compounds are called *fluorophores* or *fluorochromes.* They can absorb energy from an incident light source and convert that energy into light of a longer wavelength and lower energy as the excited electrons return to the ground state Fluorophores are typically organic molecules with a ring structure, and each has a characteristic optimum absorption range. The time interval between absorption of energy and emission of fluorescence is very short and can be measured in nanoseconds.

Ideally, a fluorescent probe should exhibit high intensity,which can be distinguished easily from background fluorescence. It should also be stable. The two compounds most often used are fluorescein and rhodamine, usually in

the form of isothiocyanates, because these can be readily coupled with antigen or antibody. Fluorescein absorbs maximally at 490 to 495 nm and emits a green color at 517 to 520 nm. It has a high intensity, good photostability,and a high quantum yield. Tetramethylrhodamine absorbs at 550 nm and emits red light at 580 to 585 nm. Because their absorbance and emission patterns differ, fluorescein and

rhodamine can be used together. Other compounds used are phycoerythrin, europium (β-naphthyl trifluoroacetone),and lucifer yellow VS.1,5Fluorescent tags or labels were first used for histochemical localization of antigen in tissues. This technique is called

**immunofluorescent assay (IFA),** a term restricted to qualitative observations involving the use of a fluorescence microscope. In this manner, many types of antigens can be detected either in fixed tissue sections or in live cell suspensions

with a high degree of sensitivity and specificity.The presence of a specific antigen is determined by the appearance of localized color against a dark background.

This method is used for rapid identification of microorganisms in cell culture or infected tissue, tumor-specific antigens on neoplastic tissue, transplantation antigens, and CD antigens on T and B cells through the use of cell flow cytometry.

**Direct Immunofluorescent Assays**

Fluorescent staining can be categorized as direct or indirect,depending on whether the original antibody has a fluorescent tag attached. In a

 **direct immunofluorescentassay,**

1. antibody that is conjugated with a fluorescent tag is added directly to unknown antigen that is fixed to a microscope slide.
2. 2- After incubation and a wash step,
3. the slide is read using a fluorescence microscope.
4. Antigens are typically visualized as bright apple green or orange-yellow objects against a dark background. Direct immunofluorescent assay

is best suited to antigen detection in tissue or body fluids, while indirect assays can be used for both antigen and this method include *Legionella pneumophila,Pneumocystiscarinii, Chlamydia trachomatis,* and respiratory syncytial virus (RSV) **(Fig. 1)**.

**Indirect Immunofluorescent Assays**

**Indirect immunofluorescent assays** involve two steps,

1. the first of which is incubation of patient serum with a known antigen attached to a solid phase.
2. The slide is washed
3. And then an antihuman immunoglobulin containing a fluorescent tag is added. This combines with the first antibody to form a sandwich, which localizes the fluorescence. In this manner, one antibody conjugate can be used for many different types of reactions, eliminating the need for umerous

purified, labeled reagent antibodies. Indirect assays result in increased staining, because multiple molecules can bind to each primary molecule, thus making this a more sensitive technique. Such assays are especially useful in antibody identification and have been used to detect treponema, antinuclear,chlamydial, and toxoplasma antibodies, as well as antibodies to such viruses such as herpes simplex, Epstein-Barr, and cytomegalovirus. **Figure 2** depicts the difference between the two techniques.Both techniques allow for a visual assessment of the adequacy of the specimen. This is especially helpful in testing for chlamydia and RSV antigens. Immunofluorescent assays in general.

**Other Fluorescent Immunoassays**

Quantitative fluorescent immunoassays (FIAs) can be classified as heterogeneous or homogeneous, corresponding to similar types of enzyme immunoassays. In this case,the label is fluorescent, and such a label can be applied to either antigen or antibody. Solid-phase heterogeneous fluorescent assays have been developed for the identificationof antibodies to nuclear antigen, toxoplasma antigen,rubella virus, and numerous other virus antigens.

 In addition,fluorescent assays are used to detect such important biological compounds as cortisol, progesterone, and serum thyroxine (T4).

However, many of the newer developments in fluorescent immunoassay have been related to homogeneous immunoassays. Homogeneous FIA, just like the corresponding EIAs, requires no separation procedure, so it is rapid and simple to perform. There is only one incubation step and no wash step, and usually competitive binding is involved. The basis for this technique is the change that

occurs in the fluorescent label on antigen when it binds to specific antibody. Such changes may be related to wavelength emission, rotation freedom, polarity, or dielectric strength.

There is a direct relationship between the amount of fluorescence measured and the amount of antigen in the patient sample. As binding of patient antigen increases, binding of the fluorescent analyte decreases, and hence more fluorescence

is observed.

**Advantages and Disadvantages of Fluorescent Immunoassay**

In principle, the use of fluorescence has the potential for high sensitivity and versatility. The methodology is fairly simple, and there is no need to deal with and dispose of hazardous substances. The main problem, however, has been separation of the signal on the label from auto fluorescence produced by different organic substances normally present in serum. Another difficulty encountered is the fact that nonspecific binding to substances in serum can cause quenching or diminishing of the signal and change the amount of fluorescence generated.

 

Fig1: Direct fluorescent antibody test for *Giardia* and *Cryptosporidium* in stool. Larger oval bodies are *Giardia lamblia* cysts, and the smaller round bodies are *Cryptosporidium sp.* cysts.



 Fig2: Direct versus indirect immunofluorescent assays. *(A)* Direct fluorescent assay. Solidphase

antigen fixed to a microscope slide is incubated directly with a fluorescent-labeled antibody.

The slide is washed to remove unbound antibody. If specific antigen is present in the patient sample,fluorescence will be observed.

*(B)* Indirect fluorescence. Patient antibody is reacted with specific antigen fixed to a microscope slide. A wash step is performed, and a labeled antihuman immunoglobulin is added. After a second wash step to remove any uncombined anti-immunoglobulin, fluorescence of the sample is determined. The amount of fluorescence is directly in proportion to the amount of patient antibody present.