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Fluorescent Antibody Test- An Effective Diagnostic Technique

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History

Immunofluorescence microscopy (fluorescent antibody) was reported by Coons, Creech and Jones in 1941. In 1942, Coons and Kaplan reported that fluorescence dyes can be conjugated with antibodies. These labelled antibodies further used as probes to detect and locate antigen specific to this antibody. Coons and Kalpan in 1950 used Fluorescence microscopy for the first time. Riggs *et al* (1958) observed that more stable Fluorochrome was fluorescein isothiocyanate (FITC). In 1958, Goldwasser and Kissling used Fluorescence microscopy for the diagnosis of Rabies.

Definition

When immunofluorescence is specifically used to detect an antigen by conjugating the fluorescent dye with the Fc region of the specific antibody (immunoglobulins) against that antigen, it is referred to as **Fluorescent Antibody Test (FAT).**

Working principal

1. The principal is based on the antigen and antibody interaction.
2. It involves the detection of antigen/protein using the specific antibodies conjugated with fluorescent dye by direct or indirect methods.
3. In the direct method of FAT, primary antibody is labelled with a fluorescent dye.
4. In the indirect method (IFAT), secondary antibody raised against the species of origin of primary antibody is labelled with fluorescent dye.

Materials required

A substance (fluorochrome) is said to fluoresce if upon absorbing light energy at a certain wavelength, it emits light of specific fluorescence which could be visualized. The most common Fluorochromes that are in use are Fluorescein Isothiocyanate (FITC) and Tetramethyl Rhodamine Isothiocyanate (RITC).

FITC (Fluoresecent Isothiocyanate) absorbs blue light and emits an intense yellow-green
fluorescence. FITC is a yellow compound that can be chemically linked to antibodies without affecting their reactivity. When radiated with invisible ultraviolet or blue light at 290 and 145 nm, FITC re-emits visible green light at 525 nm (Fig 1).

RITC (Rhodamine Isothiocyanate) absorb green-yellow light and emits red fluorescence (Fig 1).

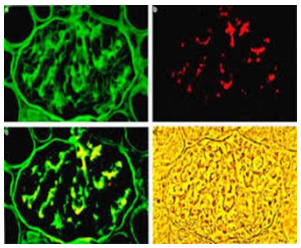

Fig 1. FITC emits yellow-green fluorescence and RITC emits red fluorescence (Belder and Granath 1973).

Conjugate: It refers to the fluorochrome tagged to the specific antibody.

Glass Slide: Impression smears of the nervous tissue are prepared on the glass slides and fixed either in chilled acetone for 4hrs to overnight or on flame or microwave oven.

Suspension: Mice brain suspensions- Infected mice brain (IMB) and normal mice brain (NMB) suspensions are prepared for use in FAT.

Fluorescence Microscope is an optical microscope that uses fluorescence instead of, or in addition to, scattering, reflection, and attenuation or absorption, to study the properties of organic or inorganic substances (James and Tanke 1991).

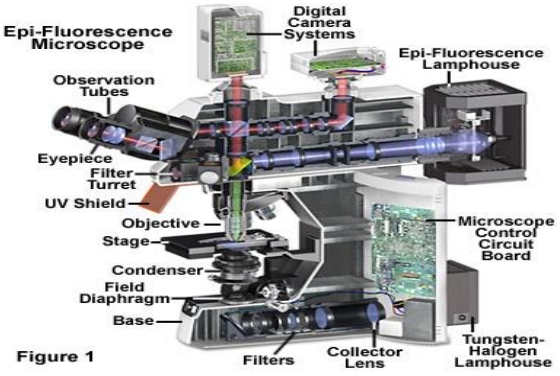

Fig 2. Rudi Rottenfusser - Zeiss Microscopy Consultant, 46 Landfall, Falmouth, Massachusetts.

The technique of immunofluorescence employs a fluorescent dye which is a substance that would fluoresce when exposed to ultraviolet (UV) rays. This principle is exploited by conjugating a specific antibody with the fluorescent dye. This conjugated dye is made to attach with the antigen. If antigen is present in the specimen being tested, then it would combine with the antibody that has already been conjugated with the fluorescent dye and that can be visualized under a microscope which is capable of emanating UV rays. When we keep such a specimen harboring the fluorescent dye under a microscope that is equipped to emanate UV rays, it would reveal the fluorescent dye.

FAT TYPES: There are mainly two types of immunofluorescence:

1. Direct immunofluorescence
2. Indirect immunofluorescence

(I) Direct FAT:

Use of a single, primary antibody, chemically linked to Fluorochrome.

This antibody recognize the target molecule (**antigen**) and binds to it.

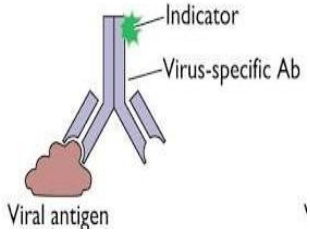

Fig 3. (Franco I.S 2009).

PROCEDURE:

1. Direct FAT is used to identify the presence of antigen in a tissue.
2. Antibodies directed against a specific antigen such as bacteria or a virus is first labelled/conjugated with FITC
3. A tissue section or smear containing the organism is fixed to a glass slide and is incubated with the FITC conjugated antibodies and then washed to remove any unbound antibody
4. When examined by dark field illumination under a microscope with an UV light source, the organisms that bind the labelled antibody will fluoresce brightly.

ADVANTAGES:

1. Shorter sample staining time as it requires rapid single step staining.
2. It is useful for the rapid diagnosis of diseases.
3. Easy to perform under suitable conditions.
4. It can be used for multiple antibodies from same host.

DISADVANTAGES:

1. Lower signal, generally higher cost which makes it unsuitable for usage.
2. Difficulties with the labeling procedure as each primary must be labeled individually.

USES:

Detection of rabies virus in brains of infected animal: (A Minimum Standard for Rabies Diagnosis in the United States) www.cdc.gov/rabies

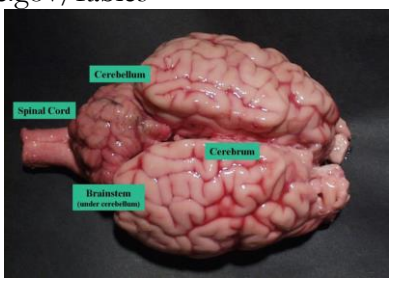

Fig 4: Dorsal view of the brain.

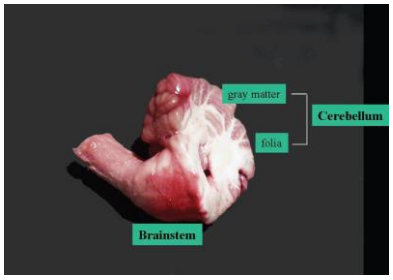

Fig 5: Surface of cut section through midbrain area showing convoluted gray matter and white foliar regions of the cerebellum, and the cerebellar connection to pons and medulla.

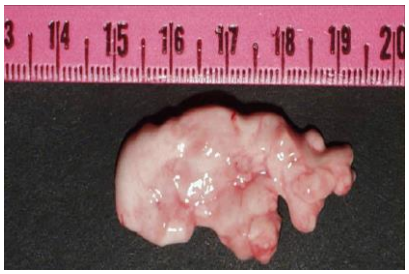

Fig 6: Dissected transverse (cross) section of brain stem.

PROCEDURE:

1. Prepare duplicate impression smears of nervous tissue by incorporating hippocampus, mid brain and cerebellum from infected and normal mice for serving as positive and negative control on either ends of a glass slide
2. Air dry the smears for about 20 mins at room temperature
3. Dip the smears in chilled acetone at -20°C and keep for 4 hrs to overnight
4. Drain off acetone and store the smears at -20°C till stained for FAT
5. Make the duplicate smears of the test sample as in the case of positive and negative controls
6. Reconstitute the lyophilised conjugate in 0.5 ml of distilled water and further dilute it with 2.5ml of distilled water to make a dilution of 1:6
7. Mix 3ml of reconstituted conjugate with 3ml of NMB and IBM separately to make a final dilution of 1:12
8. Keep the mixture of Conjugate-NMB and Conjugate-IMB in ice for 30 mins
9. Take out the positive and negative control smears out of -20°C and dry at room temperature for 10mins and mark all the smears with grease marking pencil
10. Add Conjugate-IMB mixture on one smear of the test sample and Conjugate-NMB mixtures on the other smear of the test sample. In addition, add Conjugate-NMB mixture on positive and negative control smears
11. Place the slides in a chamber with moist filter paper at the bottom. Cover the chamber and keep at 37°C for 30 mins
12. Wash the slides in 0.01M PBS twice for 5 mins each using a magnetic stirrer
13. Finally place the smears in distilled water for 5 mins
14. Air-dry the slides at room temperature and mount in 90% buffered glycerol
15. Examine the slides under microscope with a source of UV light (Dean *et al* 1996).

Others include

1. Detection of **viral, parasitic, tumor antigens** from patient specimens or monolayer of cells.
2. Used for detection of *mycobacterium avium* spp. *Paratuberculosis* in faeces, *Dichelobacter nodosus*, *listeria monocytogenes, clostridial* organisms
3. DFA tests are particularly useful for the rapid diagnosis of bacterial diseases.
4. For example, fluorescence-labeled antibodies against ***Streptococcus pyogenes*** (**group A strep**) can be used to obtain a diagnosis of **strep throat** from a throat swab (Centor *et al* 1981).

5. The diagnosis is ready in a matter of minutes, and the patient can be started on antibiotics before even leaving the clinic.
6. DFA techniques may also be used to diagnose pneumonia caused by ***Mycoplasma pneumoniae*** or ***Legionella pneumophila*** from sputum samples (Figure 7).
7. The fluorescent antibodies bind to the bacteria on a microscope slide, allowing ready detection of the bacteria using a **fluorescence microscope**.
8. Thus, the DFA technique is valuable for visualizing certain bacteria that are difficult to isolate or culture from patient samples.

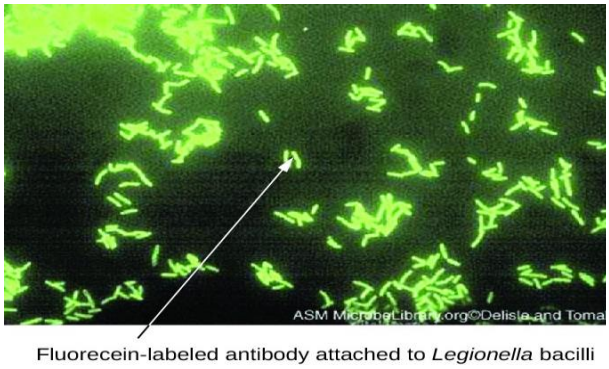

Figure 7. A green fluorescent mAb against *L. pneumophila* is used here to visualize and identify bacteria from a smear of a sample from the respiratory tract of a pneumonia patient. (Credit: modification of work by American Society for Microbiology)

A low-cost, LED based, fluorescent antibody test for the detection of the five human malaria species: (Rajasekariah *et al* 2010),

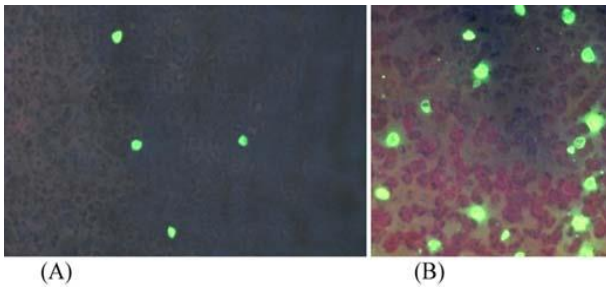

Fig 8. Pictures of iRBCs (magnification 400x) in thin smears. prepared from (A) 0.01% *P. falciparum* positive blood sample and (B) >1% P. falciparum positive sample, stained by direct IF reagent and visualized under x40 objective (400x magnification).

Direct fluorescent antibody staining of malaria parasites coupled with the use of low cost LED microscopes should increase the reliability of malaria diagnosis in resource-deprived settings and provide enhanced sensitivity, thus facilitating reliable detection of low density parasitaemias and subsequent early treatment, which may aid in minimizing emergence of drug resistance.

Laboratory Diagnosis of Human Rabies: Recent Advances: (Mani and Madhusudana 2013).

It involves demonstration of the rabies virus nucleoprotein antigen (N) in fresh brain smears of a suspected rabies case by using immunofluorescence technique

It can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis.

The specificity and sensitivity of the test almost approach 99% in an experienced laboratory and results are available within a few hours.

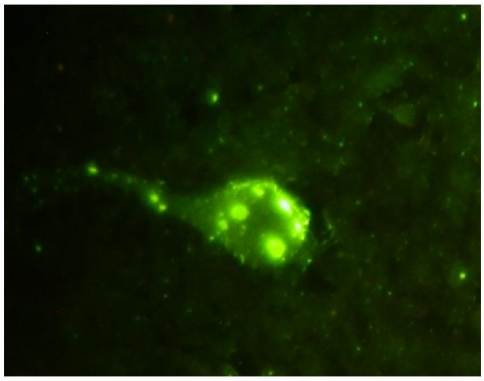

Fig 9. Fluorescent antibody technique (FAT) on human brain smear positive for rabies (Mani and Madhusudana 2013).

(II) Indirect FAT:

Indirect FAT uses two antibodies; the unlabelled first (primary) antibody and secondary labelled antibody. This secondary antibody, which carries fluorochrome, recognize the primary antibody and binds to it.

Multiple secondary antibodies can bind to primary antibody, thus has the advantage of a brighter fluorescence. It can be used to measure antibodies in the serum or to identify specific antigens in tissues or cell cultures (Madigan *et al* 2018).

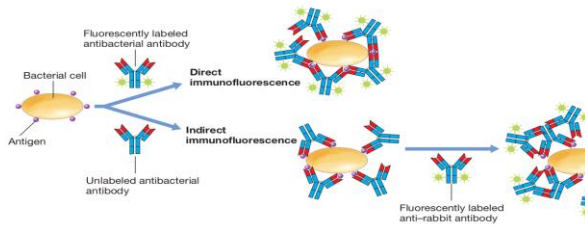

Fig 10. Direct and indirect immunofluorescence for the detection of antigen (Image source: Brock Biology of Microorganisms)

PROCEDURE:

1. Antigen is employed as a tissue smear, section or cell culture on a slide or cover slip.
2. This is incubated in serum suspected of containing Antibodies to that Antigen.
3. The serum is then washed off leaving only specific Antibodies bound to the antigen.
4. These bound antibodies may then be visualized by incubating the smear in FITC labeled antiglobulin
5. When the unbound labeled antiglobulin is removed by washing and the slide examined, the presence of fluorescence indicated that antibody was present in the test.
6. The quantity of antibody in the test serum may be estimated by examining increasing dilutions of serum on different antigen preparations.

ADVANTAGES:

1. Highly senstive and specific. A few labeled secondaries can detect many primaries.
2. Since several labeled antiglobulin molecules will bind to each antibody molecule, the fluorescence will be considerably brighter
3. Because the antiglobulins used are specific for each immunoglobulin class, the class of specific antibody may also be determined
4. Commercially produced secondary antibodies are relatively inexpensive, available in an array of colors, and quality controlled
5. The indirect fluorescent antibody test has two advantages over the direct technique
6. Since several labeled antiglobulin molecules will bind to each antibody molecule, the fluorescence will be considerably brighter than the direct test
7. Because the antiglobulins used are specific for each immunoglobulin class, the class of specific antibody may also be determined

DISADVANTAGES:

1. Need to find primary antibodies.
2. It is a two- step staining method. Hence, complex in nature.
3. Time consuming and complex as it requires antibodies from different hosts.
4. Coupling of label to primary antibody may affect antibody's ability to bind to target protein/ antigen.

USES:

1. It is often used to detect autoantibodies.
2. Commonly used in the detection of anti-nuclear antibodies (ANA) found in the serum of patients with SLE (Systemic lupus Erythematosus). (Shu *et al* 1977).
3. The indirect fluorescent antibody test (IFAT) is available for bovine *Babesia* and for *B. bigemina* (Mosqueda *et al* 2012).
4. Toxoplasmosis is confirmed serologically by the Sabin–Feldman dye test, indirect fluorescent antibody test (Fletcher 1965).
5. Antibodies to intrinsic factor and parietal cells can be detected in the serum of most patients with pernicious anemia. Various immunoassays can detect intrinsic factor-blocking antibodies; parietal cell antibodies can be detected by indirect fluorescent antibody techniques .

Recent Advances in Fluorescent Labeling Techniques for Fluorescence Microscopy: (Suzuki *et al* 2007).

1. Tremendous progress in recent computer-controlled systems for fluorescence and laser-confocal microscopy has provided us with powerful tools to visualize and analyze molecular events in the cells.
2. Fluorescent proteins such as green fluorescent protein (GFP) allow us to directly label particular proteins of interest in living cells.
3. The development of techniques of fluorescent protein (FP) labeling and

recent computer-controlled systems for fluorescence and laser-confocal microscopes has also boosted our eagerness to see directly the behavior of particular proteins of interest in living cells .

4. Fluorescent Staining Techniques Using the Fluorescent-labeled Molecular Probes to Detect the Particular Biomolecules, Structures, or Molecular Events in the Cell.
5. Zenon method is a modification of the direct method, and has a wide range of applications especially for multi-color labeling.
6. Chemical fluorescent probes are easy to use and are best suited for counter-staining. Cell permeable fluorescent dyes such as Hoechst dyes are also useful for live-cell imaging

CONCLUSION:

1. Technique that utilizes fluorescent-labeled antibodies to detect specific target antigens.
2. Fluorescence microscopy is used in medicine, environmental studies, food sanitation, biological research, education and industry.
3. The advantages of fluorescence microscopy are due to its sensitivity, specificity, adaptability and easy use.

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