Swayam Course - Analytical Techniques

Week: 7, Module 17 - Immunohistochemistry (IHC) and Immunofluorescence

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INTRODUCTION:

An increasing percentage of diagnosis in pathology is finalized to the identification of specific proteins, relevant for the patient's management, via antibodies and the deposition of a pigment or fluorochrome at the protein's location in the tissue section. The terms Immunohistochemistry and immunofluorescence are often used interchangeably. They are however clearly distinct from each another and it is important that they are used appropriately to avoid confusion. The prefix, "immuno", refers specifically to the binding of an antibody to an antigen, while the suffix confers different meanings. As the name indicates immunohistochemistry and immunofluorescence involve the binding of an antibody to a cellular or tissue antigen of interest and then visualisation of the bound product by fluorescence or chromogen detection system. Immunofluorescence has traditionally relied on the use of secondary antibodies which have been labelled with fluorescent dyes or fluorescent proteins, however labelled secondary antibodies can be a common source of unwanted background signal. Both of these techniques are widely used in the basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Now a day's, these techniques are also extensively used for the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death. In this module we focus on to understand the Immunohistochemistry (IHC) and Immunofluorescence (IF) under following objectives:

OBJECTIVES:

1. To understand the principles of IHC
2. Overview of the basic procedure of IHC
3. To understand the principles of IF
4. Overview of the basic procedure of IF

5. To discuss the advantages and disadvantages of these different techniques
6. To know the applications of IHC and IF

Principle of Immunohistochemistry (IHC):

IHC involves the process of selectively imaging antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues samples. IHC takes its name from the roots "immuno", in reference to antibodies used in the procedure, and "histo," meaning tissue. This procedure is conceptualized and first implemented to develop immunofluorescent techniques for labeling antibodies by Prof. Albert Coons in 1941. Prof. Albert Coons is an American physician, pathologist, and immunologist.

Overview of the basic procedure of IHC: There are mainly four basic steps involved in IHC technique and it takes about 2-3 hours to completely execute it.

1. **Fixation**
2. **Antigen Retrieval:**
3. **Blocking**
4. **Antibody Labelling and Visualization**

The details methodology is mentioned below:

1. **Fixation**: This step is very mandatory and basically is used to keep the tissue section/sample in place. It maintains the tissue morphology and retains its antigenicity. This step is dependent on the type of tissue being analysed, if one wants a good antigen expression one can opt for snap frozen and acetone-fixed. This can be done when an individual doesn't pay much need to the physicality of the tissue section. But, for morphology of the tissue, one must go for the formalin-fixed and paraffin-embedded (FFPE) method.
2. **Antigen Retrieval:** This is a step merely for making the antigen available for detection. For the FFPE method one should consider pre-treatment with antigen retrieval agents, which enhance the expression of the antigens of the samples by degrading the formalin induced antigen cross-linking, re-exposing epitopes on the antigen to antibody affinity accordingly. The most popular retrievals employed are the heat and enzyme retrievals, inclusive of the Heat Induced Epitope Retrieval (HIER) respectively, viz., based on heating the slides under study in buffer at a pH equivalent to 6 or 9 that is antibody dependent utilising a microwave or a pressure cooker. Some proteolytic enzymes, for instance, the pepsin or pronase can also

be used in order to break down the cross-linking chains and subject the epitopes to the antibodies.

3. **Blocking:** A step which is executed in order to reduce negative signals (false positive results). For IHC, two major factors which need to be kept in mind when blocking is, first, it should be clear, whether to simply block or the endogenous enzymes are to be inactivated in entirety in the tissues which can further activate substrates which may be used later in seeing the antibody affinity, usually done using alkaline phosphatase and endogenous peroxidise respectively. Endogenous peroxidase is generally problem for kidney/liver tissues while, alkaline phosphatise is an apex trouble in the intestine and the placenta tissues. Secondly, one should be known of the endogenous antibodies like, those on the surface of B lymphocytes in immune tissue where the secondary antibodies may cross link themselves, in turn, leading to high background staining. The selection of secondary antibodies must be done carefully.
4. **Antibody Labelling and Visualization**: For IHC technique, staining can be done in two ways:
    A. Indirect labelling method,
    B. Direct labelling method

A. Indirect labelling method: In indirect labelling, secondary antibody is covalently bound with a label and this then binds to the primary antibody during the staining procedure. **Figure-1A, B,** explains the modus operandi of this assay very lucidly.

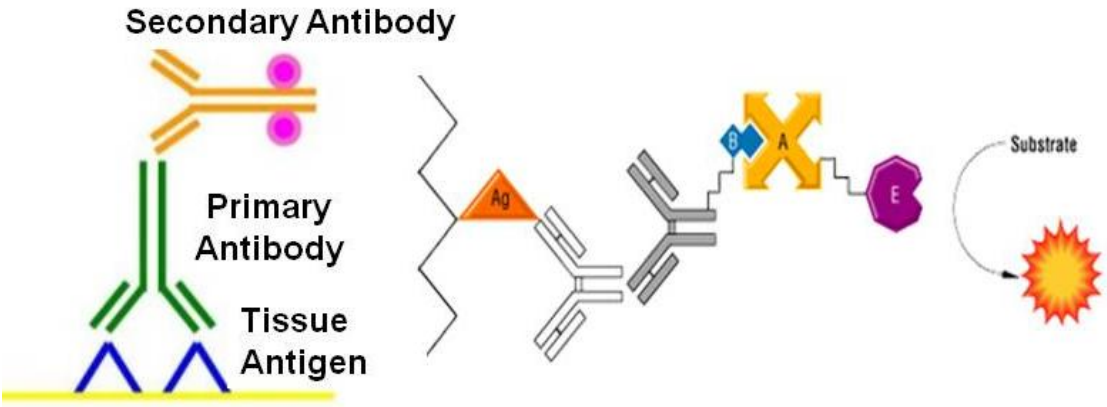

Figure-1A:Two-step Indirect method

The initial step is to incubate the primary antibody for about an hour on the tissue sample of interest, which allows the antibody to attach to the antigen. Once the

binding takes place, washing is done to remove the unnecessary and unbound primary antibody.

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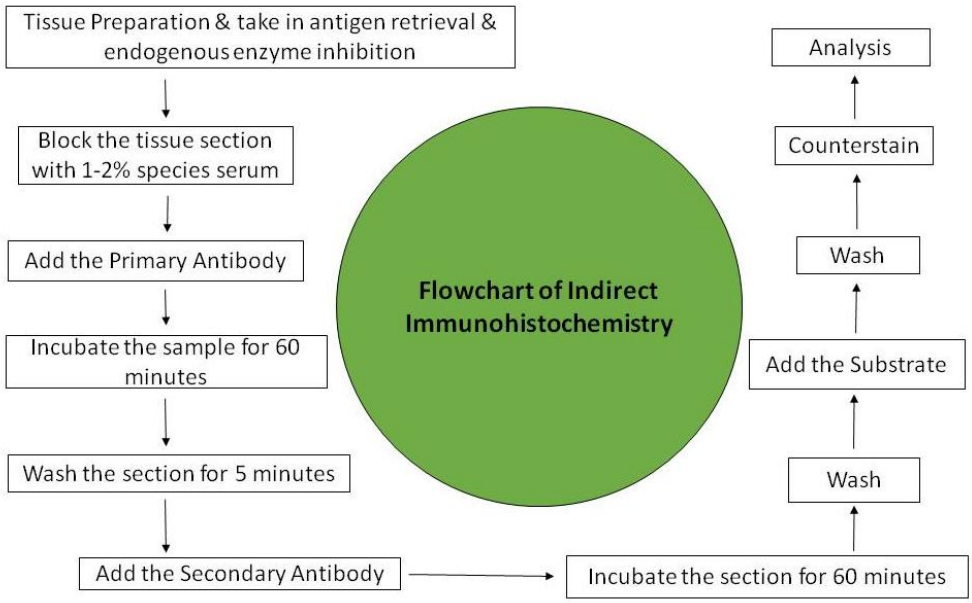


Figure-1B: Indirect IHC assay and stepwise procedure of indirect staining.

Direct Method: It is rapid and a simpler method as compared to its counterpart since here, the label is latched upon the primary antibody via a direct covalent bond, which refers to the fact that one a single incubation and washing procedure is required.

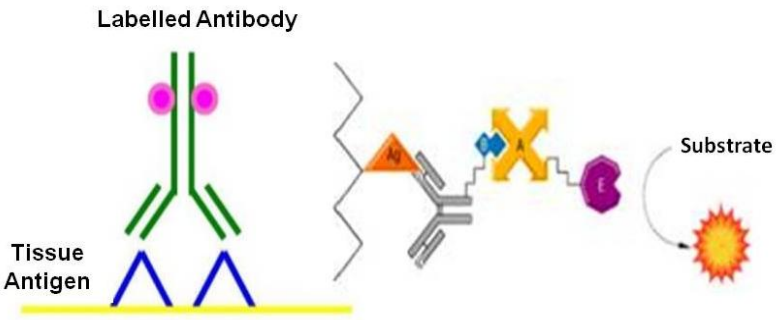


Figure-2A: Direct IHC method

This method of staining had more pros when compared to indirect staining such as, reduced assay variability, this assay is user and pocket friendly and time friendly as well. The process starts with the labelling of the samples of interest with fluorescent tags. Then, the samples are observed directly under a fluorescence microscope. But, if one has chosen the enzyme label (Horseradish peroxidise), incubation period will be required for a longer run, and in such a scenario, the enzyme acts on the substrate to develop an insoluble coloured constituent viz., localized at the region of antibody binding as shown in **Figure-2A, B**.

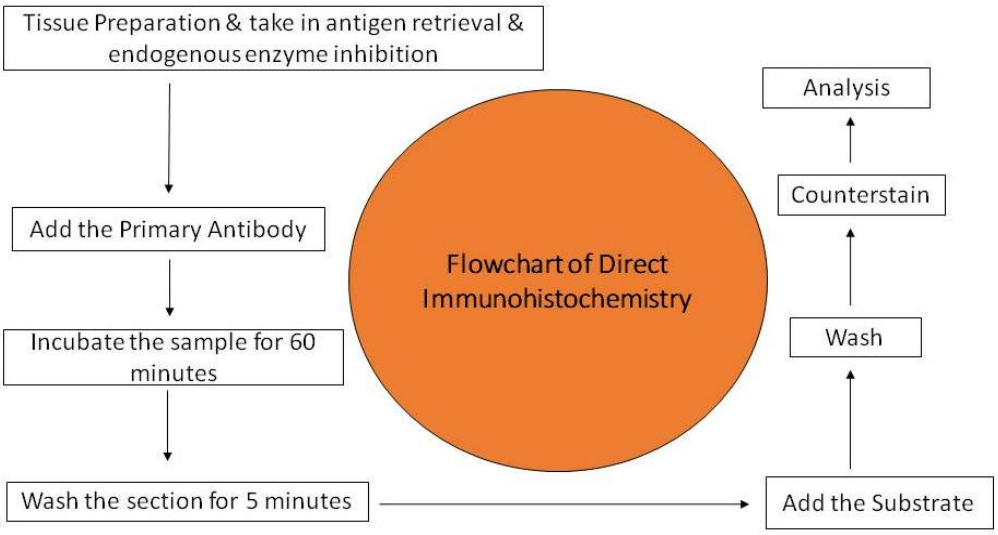


Figure-2B: Direct IHC assay and stepwise procedure of direct staining.

Advantages and Disadvantages of sub-types of IHC: The pros and cons of both the indirect and direct method of immunohistochemistry (IHC) are presented point-wise below in **Table-1**.

Method	Advantages	Disadvantages
Direct	• Fastest method • Less number of reagents required • Inexpensive • Non-specific affinity is eliminated • No cross-linking formed • Dual staining is straightforward	• Little signal amplification • Availability of directly conjugated antibodies for IHC staining is restricted
Indirect	• A small number of standard conjugated secondary antibodies is required • Commonly used technique	• Non-specific binding may occur • Extra incubation and wash steps required • Dual staining difficult to achieve

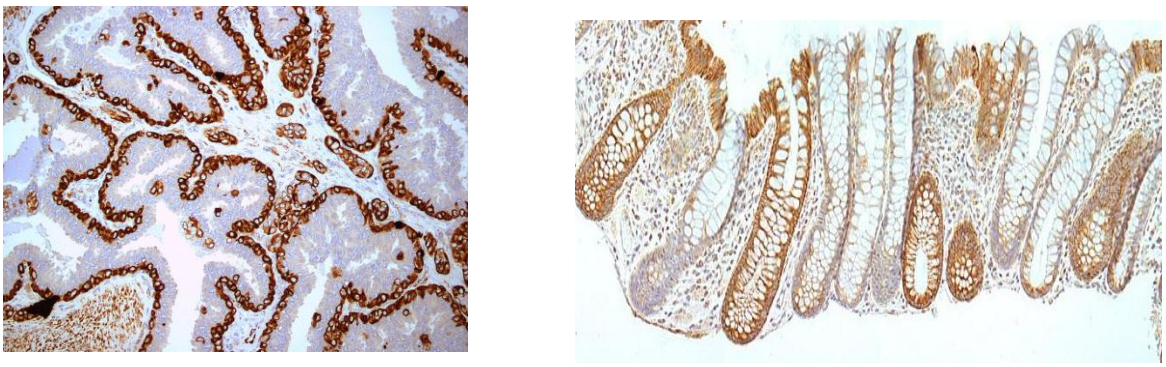
Table1. A comparison between the direct and the indirect approaches of IHC

5. Common Diagnostic IHC Markers:

The myriad variety of IHC indicators used in diagnostics analyses is very limited unfortunately. Medical fraternity and research institutes have a variety of antibodies which are used as diagnostic, prognostic and indicative biomarkers for many diseases. Some of the generally used biomarkers are as follows:

1. **BrdU**: Emloyed to determine multiplication of and to identify tumours.
2. **Cytokeratins**: Utilised for rectifying carcinomas and some sarcomas.
3. **CD15 and CD30**: For Hodgkin's disease.
4. **Alpha fetoprotein**: For yolk sac tumours and hepatocellular carcinoma.
5. **CD117 (KIT)**: Used for gastrointestinal stromal tumours (GIST) and mast cell tumors (MCT).
6. **CD10 (CALLA)**: Employed for renal cell carcinoma and acute lymphoblastic leukemia.
7. **Prostate specific antigen (PSA)**: Used for prostate cancer.
8. **Estrogens and progesterone receptor (ER & PR)**: For breast and gynaecologic tumours along with biomarkers in breast cancer and estrogens receptor.
9. **CD20**: For determination of B-cell lymphomas.
10. **CD3**: For T-cell lymphomas.

Examples of IHC staining:



A **B**

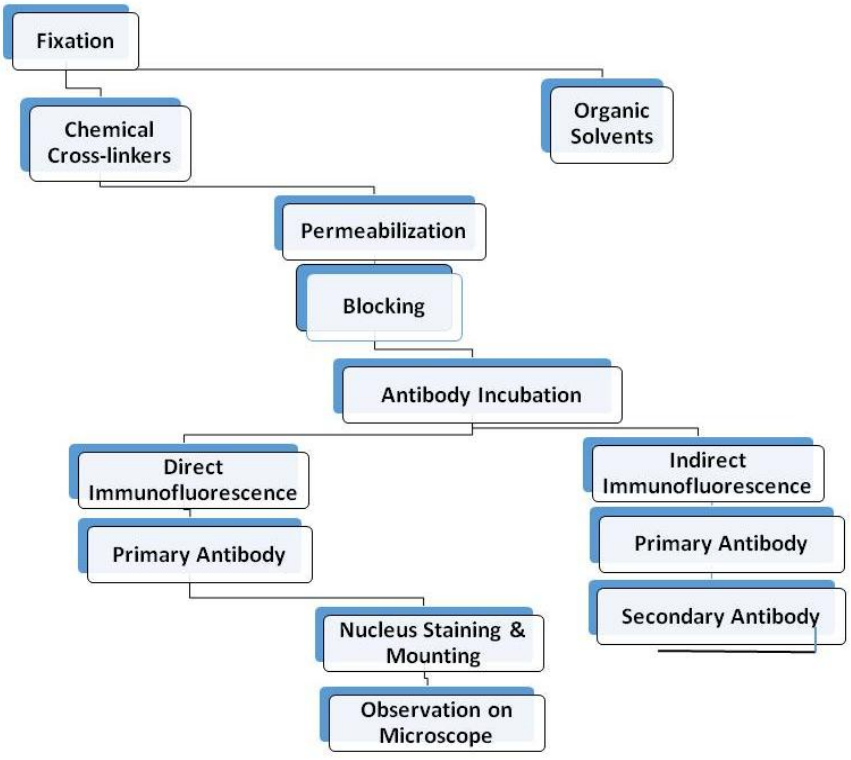
Immunohistochemical staining for A. Smooth Muscle Actin in breast and B. Cytochrome C oxidase (brown-orange) expression in Colonic crypts

Immunofluorescence (IF):

Introduction: Albert Hewett Coons was the first person to conceptualize and develop immunofluorescent techniques for labelling antibodies in the early 1940s. The main aim of this technique is to aid in visualization of the distribution of the target molecule throughout the sample of interest. The specific region an antibody which determines an antigen is called an epitope. There has have been much influential work in the domain of epitope mapping as many antibodies can easily bind on the same epitope. The affinity of the fluorophores (fluorescent tag) to the antibody itself cannot disturb the immunological specificity of the antibody or its counterpart antigen [5]. This approach is commonly studied as an example of immunostaining and is often confused with IHC and ICC respectively; it is all about making use of fluorophores to observe the region of residence of the antibodies.

Principle of IF: It is an analytical technique used in amalgamation with light and fluorescence microscopy employed for microbiological samples and this utilises specificity of antibodies to their specific antigen to target fluorescent reagents to particular biological targets within a cell.

Flowchart of immunofluorescent Experiment:

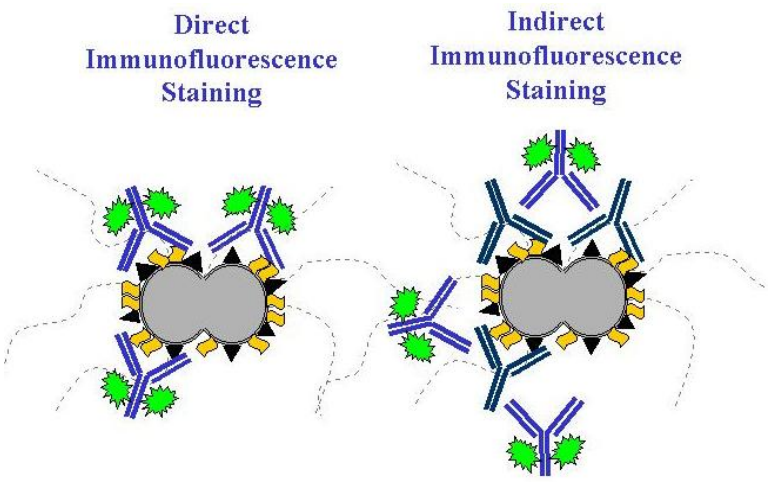


Steps Involved: There are in total following steps are required for achieving a successful IF study which are aforementioned:

1. **Designing the Experiment:** Addition of appropriate controls is mandatory to affirm that the alterations between samples are in the experimental variable(s), and that the reagents – including antibodies – are performing as expected. Such standards could encapsulate pharmacological remedies, summation of extracellular legends to regulate the signalling pathways, or comparison of cells with differential gene expression (DGE). Usually, the variables and standards are performed in parallel fashion so that the fixation step and subsequent processing can be performed simultaneously. The experiment is executed based on the type of standard being used.
2. **Preparing the Cells/Tissues of Interest:** Optimizing the cell density and cell health for initiating the immunofluorescence experiment. Tissue sections are prepared either by rapid freezing in optimal cutting temperature (OCT) medium (IF-F). It can also be prepared by dipping in paraffin (IHC-P). Once done with that, the frozen tissue samples must be slightly sectioned using a cryostat and dried for about 10 minutes before fixing the sample.
3. **Fixation:** To stabilize and preserve the samples. The best fixation saves a “life-like” picture as it halts the cataclysmic procedure of autolysis by holding the endogenous enzymes. Commonly employed fixatives are aldehyde-based fixatives such as formaldehyde, formalin and glutaraldehyde. In case of tissue samples which have been frozen fresh and cryostat sectioned should be preserved by fixing with the transcardial perfusion, post-fixation, and cryo-preservation steps, followed by freezing and sectioning. For cells, the samples should be rapidly fixed such that cellular structures and target localization are well preserved, also helping the antibodies to identify and report their targets. This is achieved by rapidly swapping the media for fixative solution.
4. **Permeabilization:** Cross linking fixatives usually don’t make the targets accessible to their antibodies, thus, Permeabilization should be executed immediately after cross linking fixation.
5. **Blocking:** This step decreases the background signal caused by non-specific binding of primary and secondary antibodies to irrelevant sites. The blocking reagent occupies “sticky-specific” sites in the sample of interest, without

disturbing with primary/secondary antibody determination of target epitopes and increasing signal-to-noise ratio (S/N).

6. **Immunostaining:** It is generally recommended to utilise an antibody at its dilution so as to derive a high signal-to-noise ratio (S/N). If the antibody is attached at lowest concentration, the fluorescence signal will be too vague and low, which in turn can make it difficult to differentiate from the background. However, a high concentration will give a dark and a prominent signal as compared to the background, viz., easily comparable. Second complementary step which is very important is immediate washing after fixation. Washing the sample at least three times for 5 minutes in PBS aids to remove unbound antibodies in order to prevent unwanted formations of primary-secondary immune complexes. For IF technique, staining can be done in two ways as we discussed in IHC: A. Direct IF staining, and, B. Indirect IF staining



7. **Counterstaining & Mounting the Sample:** DAPI is traditionally the most popular fluorescent nuclear counter stain used for IF and is an optional step. The prepared sample is then placed on to a glass slide with the help of a mounting media and is sealed with nail polish to prevent desiccation.

8. **Observation under the Microscope:** The microscope parameters such as the exposure time, excitation intensity, camera gain etc., must be checked and validated before observing the sample for a good microscopic experience. Calibration of the parameters is an important step so that the signal is in the dynamic range of the camera and pixels accurate, maintaining a high S/N ratio.

Advantages and Disadvantages of sub-types of IF:

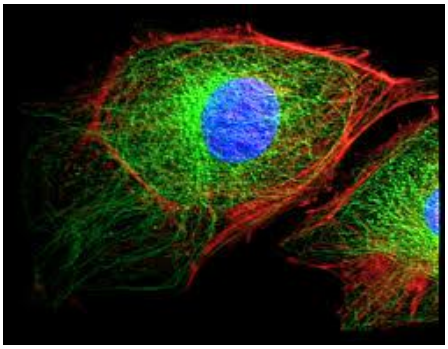
Feature	Direct	Indirect
Time	Procedure is short and easy, requires a single labelling step.	Many steps are involved and is a time taking process.
Cost	Expensive method.	Inexpensive method.
Complexity	Easy	High when compared to its counter method.
Flexibility	Restrictions in using commercially available conjugated primary antibodies restrain flexibility.	The possibility of using different conjugated secondary antibodies adds greater flexibility.
Sensitivity	Weak	Strong
Species cross-linking	Minimal	Higher possibilities
Background	Low background	High background

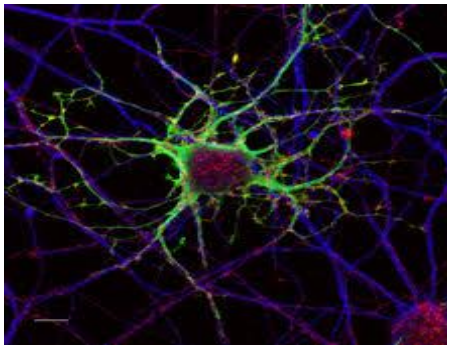
Table-1: Comparison between the direct and indirect IF methods

Common IF Diagnostic Markers:

1. **NMDA (N-methyl-D aspartate)** – Autoimmune Encephalopathies
2. **CASPR2 (Contactin-associated protein 2)** - Autoimmune Encephalopathies
3. **LGII (Leucine-rich glioma-inactivated protein I**) - Autoimmune Encephalopathies
4. **PLA2R Phospholipase A2 receptors** – idiopathic Membranous Nephropathy
5. **Anti-CUZDI and Anti-GP2** - Crohn's Disease

Examples of IF staining:





A **B**

Figure-A. Showing tubulin (green), actin (red) and nucleus (blue) respectively.

B. Primary neuronal cultures stained for Bornavirus antigens (green), Tatunus toxin (red) and tubulin (blue).