International Journal of Applied Dental Sciences 2017; 3(2): 131-134

ISSN Print: 2394-7489
ISSN Online: 2394-7497
IJADS 2017; 3(2): 131-134

www.oraljournal.com
Received: 21-02-2017
Accepted: 22-03-2017

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Immunofluorescence- An advanced diagnostic tool in dentistry

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Abstract
Immunohistochemistry or IHC refers to the process of localizing proteins in cells of a tissue exploiting the principle of specific Ag-Ab binding. It takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue. To demonstrate an Ag or Ab in a histologic section, one of the reactant (usually Ab) may be labeled with a substance such as: Radioisotope, Fluorochrome & Enzyme. The label then may be detected after formation of an immune complex (Ag-Ab). Although radioisotopes can be detected in very small amounts, their use in histology require: Auto-radiographic procedure (time consuming), may produce poor results. However, immunofluorescence & immunoenzymic procedures produce excellent localization of Ag-Ab reaction. The following article presents a review on the role of Immunofluorescence and its varied clinical applications.

Keywords: Immunofluorescence, diagnosis, dentistry, staining, dye

Introduction
IF is defined as the combined histochemical and immunologic methods to pin-point specific antigen-antibody complexes formed in tissue sections or cellular smears with the reaction of the fluorochrome labeled antibody.

Fluorescence procedure: Normally, the electrons of fluorescent substances are unexcited (in "ground state").

- When they absorb energy (usually in ultraviolet range) electron enter an energy rich "excited state"
- Electrons do not stay in this excited state & while returning to ground state they release energy (usually as light of longer wave length than original excitation light).
- In fluorescence the emitted light is given off only during the time of exposure to the excitation light or for a short period of time thereafter.
- In phosphorescence emission of light persists for a considerable time after the excitation light is no longer acting.
- If fluorescence is seen in samples that have not been stained with a fluorescent dye, is termed as primary fluorescence or autofluorescence.
- Secondary fluorescence involves the use of fluorochromes.

Literature review: Coons, Creech & Jones (1941) - Described direct IF technique. Weller & Coons (1954) - Described indirect IF and sandwich technique of IF. Kohler (1975) - Technique of monoclonal antibody preparation.

Tissue preparation for primary fluorescence: Usually unfixed smears or fresh frozen cryostat sections are used for the study of primary fluorescence. Samples should not be mounted on green glass slides. Post-fixation, if desired, may be done by a brief immersion in 95% ethanol. Formalin is not usually used for post-fixation since it tends to increase the background tissue fluorescence. An exception is if 5-hydroxytryptamine is to be demonstrated since granules containing this substance fluorescence gold yellow after formalin fixation. In many cases, autofluorescence can mimic specific secondary fluorescence. This can possibly make it difficult to distinguish between the two reactions depending on the particular Fluorescent color and structure of the substances stained.

Secondary fluorescence: Secondary fluorescence is produced when substances that are not naturally fluorescent interact with a fluorochrome dye. It is a special kind of dye that can fluoresce when excited with ultraviolet light. A major advantage of secondary fluorescence technique is their ability to demonstrate low concentrations of particular components. The methods also give good contrast, and a low-power magnification can be used for screening purposes. A disadvantage of fluorescence methods is that the localization of the stained substance appears somewhat imprecise because the fluorescence is given off in all directions.

Immunofluorescence procedures: IF methods are used most frequently to demonstrate sites of immune complexes in smear or tissue samples. Localization is revealed by labeling one of the components of the staining reaction with a fluorochrome dye, and fluorescein isothiocyanate is the most commanly used dye for this purpose. The general steps for performing most IF methods include the following:

1. Preparation of an antiserum, including animal injections, bleedings, and serum characterization
2. Conjugation of the antiserum with a fluorochrome dye
3. Purification of the conjugated dye
4. Preparation and staining of the test samples

In many cases, tagged antiserums can be purchased from commercial sources. It is wise to check the purity of commercial preparations before use by immunoelectrophoresis. Commercial antiserums usually should be diluted before use. The extent of the dilution can be determined by testing a number of dilutions of the antiserum on a known positive control.

Attachment procedures in if: There are two major types of attachment procedures used in IF methods: Direct staining & indirect staining.

Direct methods: Permit direct visualization of antigens or antibodies (considered as antigens) in tissue sections by treatment of the sample with a solution containing a fluorochrome-labeled antibody that is specific for the substance to be demonstrated. Sites of attachment to the desired antigen can be visualized by using a fluorescence microscope. To perform the direct method, a, fluorochrome-labeled antibody specific for each antigen studied is needed.

Indirect IF: Indirect procedures also may be used to demonstrate either Ag or Ab localized in a tissue section. Samples are first treated with an unlabeled antibody specific for the particular Ag, followed by treatment of the samples with a fluorochrome-labeled Ab specific to the gamma globulin of the first Ab. This last reagent attaches to sites of the specific Ab that has attached to the Ag sites in tissue. The sites of tissue Ag thereby fluoresce and can be detected by fluorescence examination.

General staining procedure considerations for IF

Tissue sections: The staining procedure should be performed immediately, if possible, but usually not later than 24 hours after the sections are cut and fixed. If the staining cannot be performed immediately, store the fixed and dried sections at 0 °C or below. Sections that will be kept unstained for prolonged periods should be stored at -40° to -60ºC. Long storage of unstained sections causes unwanted autofluorescence, may produce Ag diffusion, and may lessen Ag reactivity [1].

Tissue blocks: If staining cannot be performed for very long periods of time, frozen blocks of tissue should be stored at the lowest available temperature. The frozen blocks of tissue may be cut away from the metal object disc for storage and then remounted on the disc when the sections are to be cut. When frozen blocks of tissue are stored for any length of time, precautions must be taken to avoid desiccation of the tissue and the embedding medium. Wrap the frozen tissue block in aluminum foil. Place the frozen wrapped block in a plastic cup that contains a small amount of ice. Seal the cup and store at -40° to -60 °C. Tissue blocks may be stored in this manner for months before sectioning and staining.

Staining procedure considerations for direct IF: Procedural details will vary somewhat depending on the exact stain being performed. Before beginning the procedure, encircle the tissue with a diamond marking pencil to facilitate location of the tissue after the sections are coverslipped. Rehydrate the tissue by placing the slides in 3 changes of phosphate-buffered saline over a 10 minute period at room temperature. This step facilitates subsequent staining. Remove excess fluid from the slide with cotton gauze to prevent dilution of the antiserum in the subsequent step. Remove all the fluid except that contained within the encircled area [2].

The tissue itself must not be allowed to dry from this step onward. Drying produces artifacts primarily because of salt precipitation. Place a few drops of fluorescein-conjugated monospecific antiserum directly on top of the tissue. Place the slides in a moist chamber for 30 minutes to 2 hours at room temperature. Petri dishes with dampened filter paper in the bottom of the dishes are convenient moist chambers. Wash the slides with 3 changes of phosphate-buffered saline over a 10 minute period at room temperature. Thorough washing is critical, otherwise nonspecific fluorescence will remain in the tissue and the interpretation of the slides will be confused [3, 4].

Cover slipping: Several mounting media have been used. In all cases, the mounting media should not dissolve fluorescent material and should not be fluorescent. For proper coverslipping, three methods are used commonly: Method A, B & C.

Method A: Wipe off excess saline from each slide and coverslip by using a buffered glycerol mounting medium. Gelvatrol may also be used and provides a more permanent mount than does the glycerol. Fading does occur in the slides, but storage of the slides in the dark at refrigerator temperature will prolong preservation of the slides.

Method B: Air-dry slides after the last buffer wash and mount in Fluoromount or DPX. Method C: Rinse slides in distilled water; blot dry, clear in xylene, and mount in new Unimount.

Examination of sections: Ideally, slides should be examined almost immediately after coverslipping. If this is not possible, keep the slides cold and away from light until they can be examined. Examine slides by using appropriate exciter and barrier filters. Ag- Ab complex sites appear apple green if FITC is the conjugate dye or orange if rhodamine B is the conjugate dye.

Controls for staining specificity: Staining should occur with preparations that contain the desired Ag, and staining should be located at the antigenic sites only. Appropriate known

positive controls should be included with test cases. In addition, the following controls may be used: Autofluorescence control, use of conjugated control serum, and blocking test [5].

Autofluorescence control: This is an unstained slide that has been treated like the test slide except that it has not been exposed to the antiserum conjugate. Only autofluorescent sites should be visible when viewed with the fluorescence microscope.

Blocking test: This test involves pretreating the section with unlabeled antiserum that is specific for the Ag. An Ag-Ab action should occur, and all available Ag binding sites should, be filled at this step. The second part of the procedure involves staining the treated section with the fluorescent dye-labeled specific antiserum, and, because all Ag sites have been occupied theoretically, no staining should occur. A similar section may be treated with unconjugated control antiserum that lacks the specific Ab to combine with the Ag. This pretreatment then is followed by exposure of the section to the labeled specific antiserum. Staining should occur, since theoretically no binding should have taken place with the control antiserum and the antigenic sites are free to react with the specific antiserum and thereby be visualized.

Fluorescence difficulties

Fading: All fluorescent preparations will fade upon exposure to strong light, especially ultraviolet light. The rate of fading increases with the increased intensity of irradiation; therefore, excitation at a very short wavelength should be avoided. Some workers believe that a certain amount of recovery from fading can occur if, after fluorescence examination, sections are stored in the dark at a cold temperature.

Counterstaining: Counter stains have been used to eliminate nonspecific background fluorescence and enhance cellular detail. Two useful methods for counterstaining slides, prepared by the direct and indirect IF techniques are the methyl green method and, methyl green- Eriochrome-black (chromogen black) method. The use of methyl green results in a red fluorescence of the nuclei, and there is no masking of the specific Ag-Ab fluorescence. The methyl green-Eriochrome black combination counter stain is reported to give excellent elimination of background fluorescence; however, fading occurs more quickly with this counter stain than with the methyl green alone.

Fluorescence microscope: In addition to, the usual requirements for a microscope, a special light source and special filter system are both needed for fluorescence work.

Light source: A fluorescent substance is one that, upon being excited with short-wavelength radiation (such as ultraviolet light) will emit light of a longer wavelength (Visible light).The light source needed in fluorescence work, therefore, should be one that will give off an appropriate short-wavelength light. The two major kinds of light sources used are- high pressure mercury vapor lamps and halogen lamps [6].

Mercury vapor lamp: Intense illumination with over 30% emission at 365 nm. It's efficient and convenient. Does not require water cooling Average life 200 hours Most manufacturers provide this type Good for surface staining of lymphocytes and for immunoglobulin detection Power unit and replacement burners comparatively expensive Once turned off, lamp cannot be relit until it has cooled Bleaching of fluorescence reaction occurs fairly rapidly because, of photochemical destruction of fluorochrome As lamp gets older, there tends to be a reduction in beam intensity and a resultant decrease in fluorescence excitation.

Halogen lamp: Convenient and efficient Suitable for most routine applications of fluorescence Control unit and replacement bulbs inexpensive Slow development of photolysis Lamp can be switched on and off and does not need warming-up or cooling-off periods Emits little ultraviolet light; therefore, less autofluorescence of tissue components is apparent Light emitted may not be bright enough for all IF studies.

Exciter filters: Although the light source used emits short wavelength light primarily, it does not give off such light entirely, and because of the wavelength variability, an exciter filter is needed. This filter is placed between the light source and the reflecting mirror.

Functions of exciter filter are:

1) To allow maximal transmission of the light of the desired wavelength to pass through and to subsequently excite the fluorochrome.
2) To permit only minimal transmission of undesired wavelength light.

Barrier filters: Fluorescent substances in the specimen become excited by the short-wavelength light that they receive through the exciter filter and emit longer wavelength light. There are now two kinds of light traveling towards the observer's eyes: short-wavelength light from the light source & visible light from the fluorescing specimen. The short-wavelength light (especially if it is ultraviolet light) can cause ocular damage. Therefore, another filter, called a barrier (or contrast) filter, is needed. These barrier filters are placed in suitable location behind the objective and function to: Exclude light below a certain wavelength and to allow passage of the longer wavelength light emitted by the fluorescent specimen.

Illumination systems: There are presently two systems of illumination that are effective in fluorescence microscopy: Dark-field illumination with transmitted light and Bright-field epi-illumination [7].

The dark field- transmitted type is most frequently employed, and, conventional microscopes can be converted to this type with the proper accessories. The dark-field illumination is accomplished by the use of a cardioid condenser. Enlarging the area for specimen inspection can be accomplished by interposition of a toric lens beneath the condenser. These lenses can be used over a range of objective powers, enable tissue to be inspected and photographed, with a low-power objective, and also provide good illumination for critical examination with higher power objectives (40x).Fluorescence epi-illumination is newer than the transmission type.

Conclusion: IF is an important adjunct in diagnostic procedures, and some lesions may have very characteristic IF picture. But its technique sensitivity, cost and need of set up prohibits its regular application for most of the pathologists.

Source of support: Nil

Conflict of interest: None

References

1. Theory & practice of histological techniques. Bancroft JD, Gamble M. 5th edition, 579-92.
2. Theory & practice of histological techniques. Bancroft JD, Steven A, Turner DR. 3rd edition, 451-64.
3. Cellular pathology technique. Culling CFA, Allison RT, Barr WT. 4th edition, 349-65.
4. Bienenstock J, Dolejel J. Preservation of immunofluorescence. J Histochem Cytochem. 1970; 18:518.
5. Schenk EA, Churukian CJ. Immunofluorescence counterstains. The J. of histochemistry and cytochemistry. 1974; 22:962-6.
6. Theory & practice of histotechnology. Sheehan DC, Hrapchak BB. 2nd edition, 310-19.
7. Raghu AR, Nirmala NR, Sreekumaran N. Direct immunofluorescence in oral lichen planus and oral lichenoid reactions. Quintessence Int. 2002; 33:234-9.