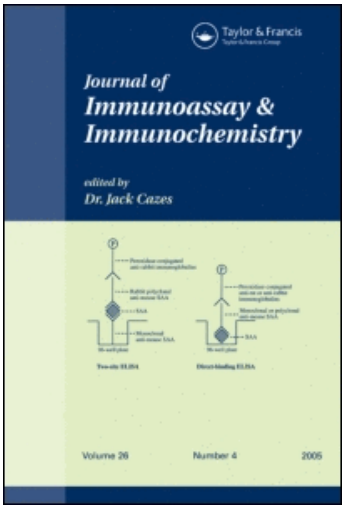

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Immunofluorescence and Enzyme Immunomicroscopy Methods

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Immunofluorescence and Enzyme Immunomicroscopy Methods

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I. INTRODUCTION

Immunohistochemistry is a simple, yet powerful, tool that is being used with ever-increased frequency and permits in rendering a confident analysis and, when used in clinical laboratory medicine, helps in making a definitive diagnosis (1–22). Two basic detection systems are used in immunohistochemistry: immunofluorescence and enzyme immunohistochemistry. Whether one chooses to use immunofluorescence or immunoenzymatic procedures, largely depends on what one is trying to achieve and on an individual's expertise and preferences. In general, both methods will provide precise and specific information, and both employ the same immunological principle of antigen–antibody interactions.

Most histopathologists, because they are used to light microscopy, may prefer immunoenzymatic techniques and not so much immunofluorescence. This is because the staining reactions with the immunoenzymatic method may be evaluated using the light microscope they use to evaluate the histochemical (hematoxylin–eosin; H&E) reactions. In addition, the relative permanence of the chromogenic reactions and the option of counterstaining enhances the preference of the enzyme immunohistochemical methods by certain pathologists. However, the presence of endogenous enzyme activities of the various antigenic substrates, the extra steps involved in substrate incubation, the loss of enzymatic reactions on storage of the enzyme-labeled conjugates and its costs, and the variable and decreased penetration of enzyme–antibody conjugates in the intact cells, all are some of the limitations of enzyme immunohistochemical methods.

Immunofluorescence, despite its simplicity, specificity, and sensitivity, as with enzyme immunohistochemistry, has its own limitations. These include that samples are less permanent, they are prone to fading on excitation and visualization under the microscope, and they require special microscopes for evaluation of the reactions. However, with the advances in laboratory medicine, some of these limitations have been minimized. From the foregoing we may conclude that both techniques are widely employed and can be used in clinical and research laboratories.

II. IMMUNOENZYMATIC METHODS

There are several immunoenzymatic-staining methods used to detect and localize antigens or antibodies. The choice of the method depends on certain variables, such as the type of the

Reprinted from *Immunochemistry*, C. J. van Oss and M. H. V. van Regenmortel, eds., Marcel Dekker, Inc., New York, 1994, 829–847.

specimen being investigated and the degree of sensitivity required. The immunoenzymatic methods use the properties of the enzyme to convert colorless chromogenic substrates into the colored end products. Of the various enzymes, horseradish peroxidase (HP), calf intestine alkaline phosphatase (AP), glucose oxidase from *Aspergillus niger*, and β-galactosidase have been employed (17–31). The various chromogenic substrates that can be used with various enzymes are summarized in Table 1. There are many excellent reviews that describe the properties of the enzymes and suggested procedures for the selection and preparation of various substrates (17–31).

A. Direct Method

In the direct method, an enzyme is conjugated to the primary antibody, which then can directly react with the antigen in the tissue. The binding of the enzyme-labeled primary antibody is subsequently detected by the use of a chromogenic substrate (Fig. 1).

B. Indirect Method

The indirect method employs a labeled secondary antibody and can involve either two or three steps. This method amplifies the signal, compared with the direct method. Similarly, the three-step method amplifies the detection signal and, hence, the sensitivity of the method in comparison with the two-step technique. The indirect methods are commonly used to detect the presence of autoantibodies in the serum.

1. Two-Step Method

In the two-step method, an unconjugated primary antibody binds to the directed antigen, followed by an enzyme-labeled secondary antibody to the primary antibody. This is followed by reaction with an appropriate chromogenic substrate (Fig. 2).

Table 1 Various Substrates and Chromogen of Use in Enzyme Immunohistochemistry

Substrate	Product
Peroxidase (M_r 40 kDa)	
3,3′-Diaminobenzidine tetrahydrochloride (DAB)	Brown
3-Amino-9-ethyl carbazole (AEC)	Red
4-Chloro-1-naphthol (CN)	Blue
p-Phenylenediamine dihydrochloride	Blue-black
Alkaline phosphatase (M_r 100 kDa)	
Fast red	Bright red
New fuchsin	Magenta red
5 Bromo-4-chloro-3-indoxyl phosphate (BCIP), nitroblue tetrazolium (NBT)	Blue-black
β-Galactosidase (M_r 500 kDa)	
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (IbGa)	Blue
Glucose oxidase (M_r 185 kDa)	
Glucose/nitroblue tetrazolium	Blue-black
Glucose/2(*p*-indophenol)-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT)	Red
Glucose/trinitroblue tetrazolium (TNBT)	Brown

The suggested procedures for the preparation and the use of the listed substrate–chromogen can be found in various text books and manuals on the subject (17).

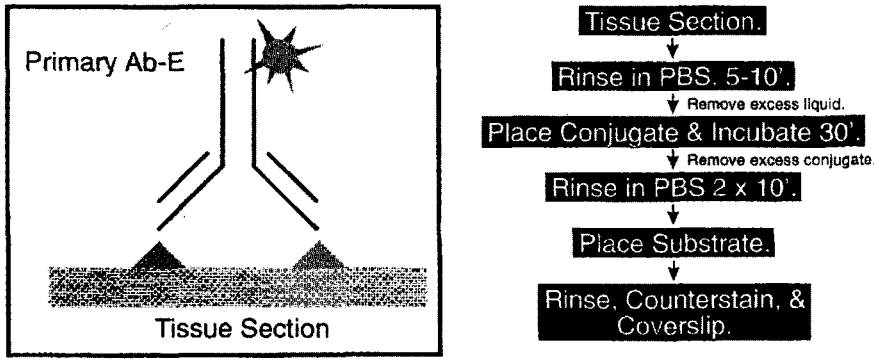


Figure 1 Direct immunoenzymatic method: In this method enzyme-labeled primary antibody reacts directly with the tissue antigen.

2. Three-Step Method

In the three-step method, primary and secondary antibodies are applied sequentially, as in the two-step one. In addition, a second enzyme-conjugated antibody layer is added. The prerequisite of this method is that both the secondary and tertiary antibodies must be conjugated to the same enzyme (Fig. 3).

The soluble enzyme–immune complex methods, such as peroxidase–antiperoxidase (PAP), alkaline phosphatase–antialkaline phosphatase (APAAP), avidin–biotin complex (most com-

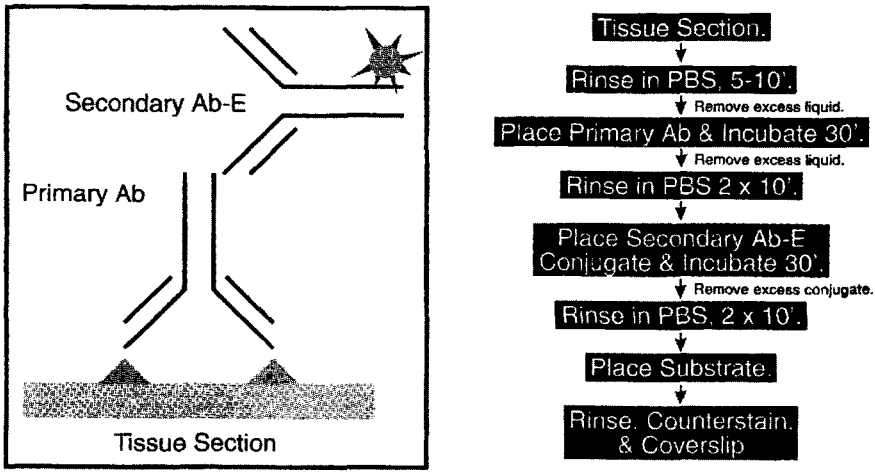


Figure 2 Two-step indirect immunoenzymatic method: Enzyme-labeled secondary antibody reacts with the primary antibody bound to the tissue antigen.

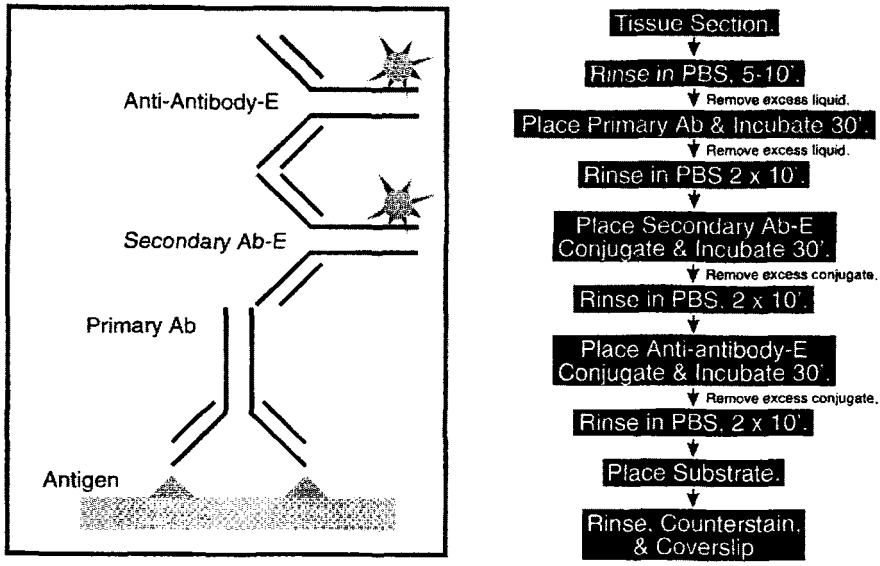


Figure 3 Three-step indirect method: This method is an extension of the two-step indirect method. In this method, labeled antiantibody reacts to the enzyme-labeled secondary antibody.

monly referred to as ABC), and labeled avidin–biotin (referred to as LAB), are variables of a three-step indirect method.

Soluble Immune Complex Method. Soluble enzyme immune complex methods employ preformed soluble enzyme–antienzyme immune complexes. The enzyme is added in excess to provide soluble complexes of the enzyme–antienzyme. The main requisite of this method is that the primary antibody and the antibody of the enzyme complex must be made in the same species, so that the secondary antibody can provide a linkage between the primary antibody and the antibody of the enzyme–immune complex. In addition, the second antibody must be added in excess, so that one of its Fab sites is free to bind the antibody of the enzyme–immune complex.

The two most commonly used enzyme–antienzyme immune complexes are peroxidase–antiperoxidase (PAP) and alkaline phosphatase–antialkaline phosphatase (APAAP). The PAP complex was first described by Sternberger in 1970 (32) and comprises three molecules of enzyme peroxidase and two molecules of antiperoxidase. The APAAP complex comprises two molecules of enzyme alkaline phosphatase and one molecule of its antibody. The presence of an increased number of enzyme molecules per antigen-binding site imparts increased sensitivity to this method. Of the soluble enzyme–immune complexes, PAP is the most commonly used, except in situations when endogenous peroxidase is present. In such an event, either the endogenous peroxidase activity is quenched by treatment of the antigen substrate with 3% hydrogen peroxide for 3–5 min, or APAAP may be considered as a replacement for the PAP method.

Avidin–biotin Method. The avidin–biotin method possesses certain advantages over PAP and APAAP methods in that (1) avidin has high-affinity constants for biotin, with dissociation constants of 10^{-19} M, compared with antibodies with dissociation constants in the range of 10^{-5}, and (2) avidin has a valency of 4 for biotin; thus, up to four molecules of biotin can bind to a

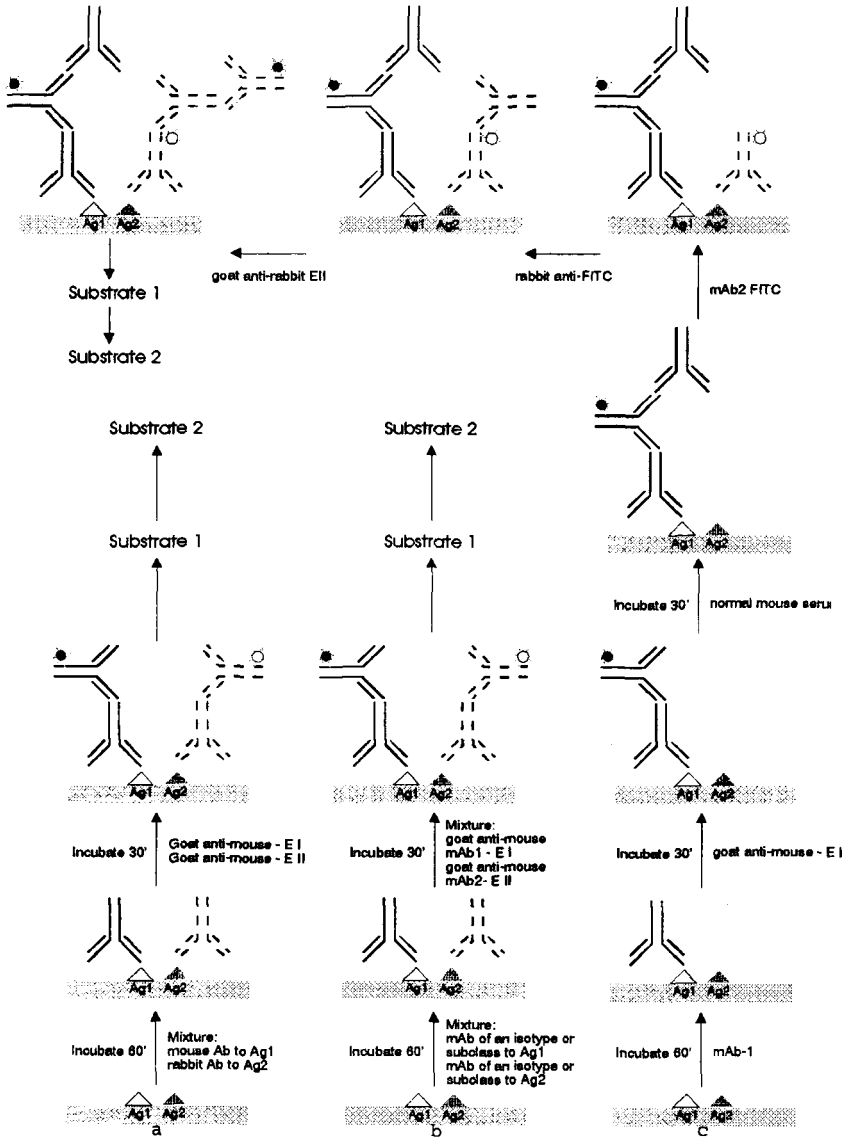


Figure 4 *Double-labeling immunoenzymatic reactions—a schematic representation:* (a) Immunoenzyme double-staining protocol using two unlabeled polyclonal primary antibodies. (b) Immunoenzyme double-staining using two unlabeled monoclonal antibodies of different isotypes or subclass. (c) Double-staining procedures using one unlabeled monoclonal antibody and one FITC conjugate monoclonal antibody.

molecule of avidin. These, as well as that many molecules of biotin can be attached to one molecule of the antibody because of its small molecular size, impart increased sensitivity to this staining method. In addition, owing to the high-binding affinity of avidin to biotin, higher dilutions of labeled antibody and short incubation times enhance the usefulness of this staining method.

C. Double Staining

There may be situations in which it will be necessary to obtain confirmation on the possible colocalization of the two antigens. This can be done by comparisons of individual immunohistochemical patterns for each of the antigens involved. However, such a method possesses certain major disadvantages: (1) certain structures may not be present on every tissue section or cell smear; (2) the size of the cell and the lack of appropriate tissue landmark may make the comparison difficult; (3) such studies may be difficult on certain preparations, such as cell smears, cell cultures, and tissue imprints. Thus, the simultaneous detection of the two antigens on the same specimen is the best alternative for colocalization of these antigens. The double-labeled staining protocol, in general, is a combination of the staining protocol used for the detection of two individual antigens (Fig. 4). The success of a good double-staining protocol lies in the selection of reagents devoid of any cross-reactions and the formation of two highly contrasting colored precipitates.

Immunoenzyme double-labeling is more than a combination of the two staining protocols for the detection of two different antigens, and many investigators consider immunoenzyme double-labeling as controversial for the colocalization of two antigens. Cross-reactivity between the two staining sequences, use of suboptimal color combinations, and the difficulties in colocalization of two antigens at varying concentrations may contribute to their reservation. The suggestions and modifications suggested by Van der Loos and co-workers (33) help optimize its use.

III. IMMUNOFLUORESCENCE

Fluorescence microscopy was first described by Albert Coons in 1941 (34) and is routinely used for studying the distribution of substances present in small amounts. In fluorescence microscopy the specimen to which fluorochrome is bound is illuminated with light of a short wavelength; part of this light is absorbed by the specimen and remitted as fluorescence at a wavelength that is longer than that of the incident light. To visualize the fluorescence under the microscope, the incident light is filtered out by a secondary set of filters, referred to as barrier filters, placed between the specimen and the eye. The basic arrangement of a fluorescence microscope is schematically shown in Fig. 5.

Fluorescence microscopy in principle is similar to the enzyme immunohistochemistry described earlier, except that a fluorophore, instead of the enzyme, is used as the label. The fluorochrome has the property that, when irradiated with incident light, also referred to as exciting light, of a certain short wavelength, the fluorophore absorbs some of the light and is excited to a single excited state. On relaxation of the excited state, fluorescence is produced by radiative transition to the resting or the ground state (Fig. 6). As the fluorochrome has the property of emitting fluorescence on excitation, it possesses advantages over enzyme immunohistochemistry in that it can be directly examined without having to develop a chromogenic reaction by incubation with the substrate. The various fluorochromes primarily used in immunofluorescence microscopy are listed in Table 2. Of these fluorescein—particularly fluorescein isothiocyanate (FITC)—is used extensively in immunofluorescence microscopy. The FITC can be coupled to the proteins—through a thiocarbamide bond to the amino groups of the proteins—by a simple

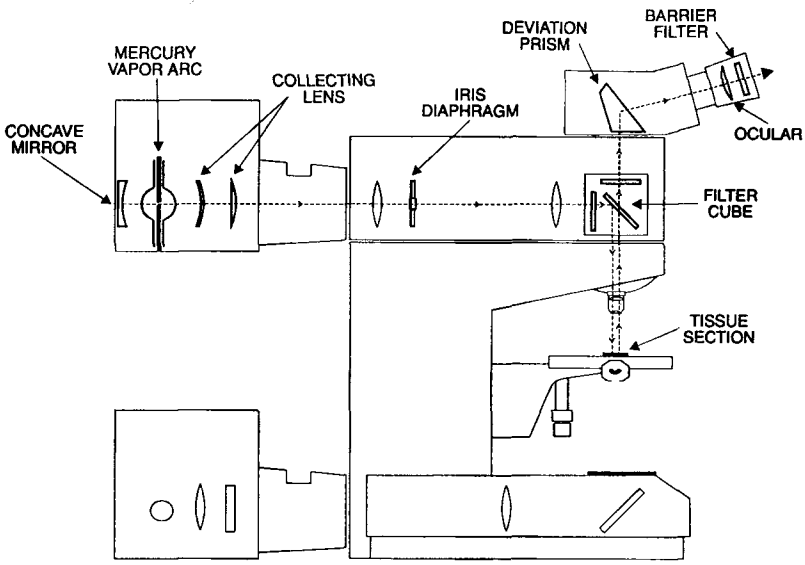


Figure 5 A schematic representation of a fluorescence microscope.

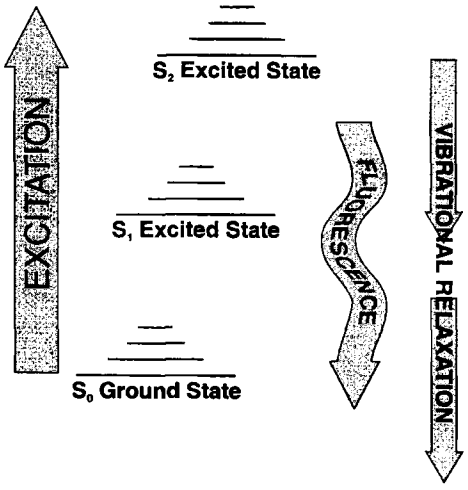


Figure 6 Schematic representation illustrating various molecular energy levels during absorption of light: Absorption of photon produces excitation from the ground state (S_0) to an excited state (S_1 or S_2). Fluorescence is produced by radiative transition of the excited state S_1 to the ground state S_0.

Table 2 The Common Fluorochromes of Use in Immunofluorescence

Fluorochrome	Excitation (nm)	Emission (nm)	Color
Fluorescein	495	525	Green
Rhodamine	552	570	Red
Texas red	596	620	Red
Phycoerythrin	480, 545, 565	578	Orange, red

coupling method. It produces green fluorescence when excited with the UV light, producing an excitation at 495 nm and emission at 525 nm (Fig. 7).

Immunofluorescence microscopy is simple and, in the clinical laboratory, it is the mainstay for detecting small amounts of most autoantibodies. Fluorescence microscopy has advantages over enzyme immunohistochemical methods in that it is useful for detecting small amounts of antigens and can be used on opaque objects. The optical sensitivity of the immunofluorescence microscope can be substantially increased by careful selection of excitation and emission filter combinations to favor the excitation and emission of the particular fluorochrome.

There are certain limitations of fluorescence microscopy in comparison with enzyme immunohistochemical methods. These disadvantages include (1) the expensive instrumentation of the required fluorescence microscope and (2) fading of the fluorescence under irradiation. The latter, however, can be minimized by the use of certain antioxidants (see Sec. III.E).

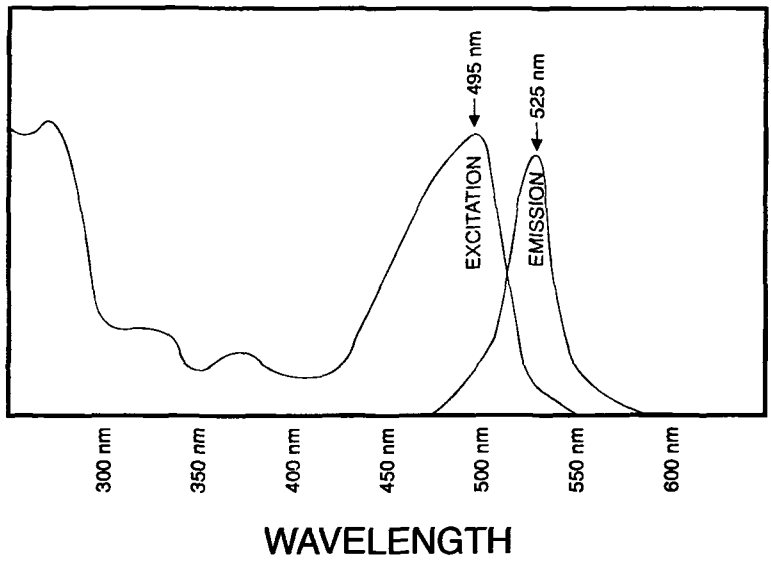


Figure 7 Excitation and emission spectra of fluorescein isothiocyanate (FITC).

A. Direct Method

The direct method is similar to the enzyme immunohistochemical method, except that the indicator tag is a fluorochrome, rather than the enzyme. Because the fluorochrome fluoresces on excitation, this is one step shorter than the enzyme immunohistochemical method. Direct immunofluorescence is used routinely for immunopathological examination of skin and kidney biopsies for the detection of in vivo bound immune deposits. Direct immunofluorescence entails the steps outlined in Fig. 8.

B. Indirect Immunofluorescence

Indirect immunofluorescence methods are used routinely for the detection of circulating antibodies in the serum of patients with various autoimmune disorders. The steps involved are similar to those described for the two-step enzyme immunohistochemical method and are briefly delineated in Fig. 9.

C. Double Immunofluorescence

The double-immunofluorescence method, even though of little use in routine testing, can be of extreme value, however, in situations for which the distribution of different antigens in one cell need to be compared. The routine double-immunofluorescence method entails the use of two or more distinct antibodies to different antigens conjugated with various fluorochromes. To observe the fluorescence in such circumstances, it is necessary to change the filters. In addition to the change of filters, the other limitation of this method is that different fields, tissue sections, or cell smears need to be examined. Such a system, even though it may be adequate in most situations, possesses certain limitations. Almahbobi and Hall (35) have recently described a modification of this double-immunofluorescence test system. In their system, four antibodies raised in three species are used in detecting two different antigens and requires that one primary antibody and one of the secondary antibodies be raised in the same species. Because the two secondary antibodies are conjugated to two different fluorochromes, both antigens could be visualized with one light filter, while one antigen is visualized with another filter. Such a method, as depicted in Fig. 10, allows more efficient comparison of the distribution of two antigens in a single field.

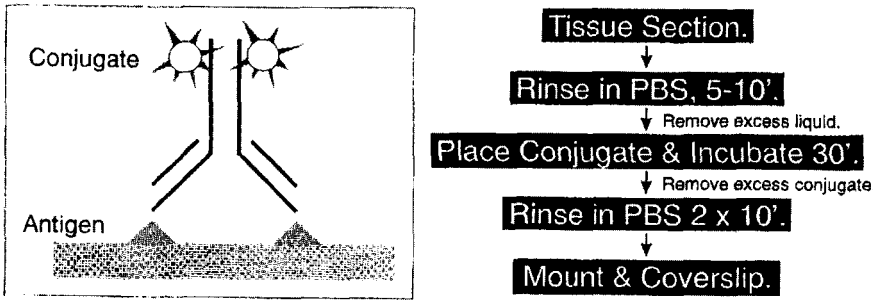


Figure 8 Direct immunofluorescence: The fluorochrome-labeled conjugate binds directly to the tissue antigen.

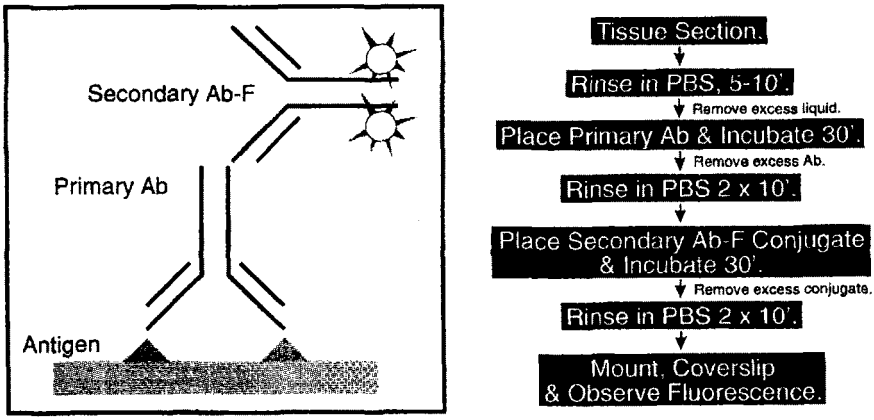


Figure 9 Indirect immunofluorescence method: In this the fluorochrome-labeled, rather than the enzyme-labeled, secondary antibody binds to the primary antibody, which has already been bound to the antigenic substrate.

With the fluorochrome system described in Fig. 10 and with the blue filter, antigen 1 will fluoresce yellow, which will be distinct from the green fluoresce of antigen 2. Such a method thereby precludes the use of a second filter for the study of co-localization of two antigens.

D. Mounting Medium

There are primarily two kind of mounting medium employed in immunofluorescent staining (2). These mounting media can also be used in most of the immunoenzymatic-staining reactions. The first is buffered glycerin—made by mixing nine parts of the glycerin with one part of the buffer, which is usually phosphate buffered saline (PBS) pH 7.4. The disadvantage of this mounting medium is that the staining undergoes rapid photobleaching on excitation; additionally, the stained slides mounted in this medium cannot be stored for prolonged periods. The degree of photobleaching depends, in part, on the pH of the mounting medium and, in part, on the viscosity of the preparation.

The other mounting medium used is referred to as semipermanent polyvinyl alcohol-based medium. This medium is useful in preserving the immunofluorescence reactions for extended periods (36). The medium can be prepared by mixing 20 g of polyvinyl alcohol (Gelvatol 20-30, Monsanto) with 80 ml of PBS for 16–24 h at room temperature. Once in solution, 40 ml of glycerol is added and the mixture stirred for another 16–24 h. The preparation is then centrifuged and the pH adjusted to 7.2.

E. Photobleaching

One of the characteristics and the limitations of fluorescent microscopy is that fluorescent preparations nearly always fade on irradiation. The mechanism of photobleaching is not entirely known. The various factors that may explain photobleaching include photochemical decomposition, which may include oxidative and nonoxidative mechanisms (37,38). The rate of photobleaching is characteristic of the fluorochrome. Some undergo rapid photobleaching, whereas

others are relatively stable. The rate of photobleaching is dependent on the intensity of irradiation. It can be diminished if the specimen is mounted in a medium containing antioxidants, such dithiothreitol, *p*-phenylenediamine, *n*-propyl gallate, or β-mercaptoethanol. The efficacy of these compounds in preventing photobleaching varies, depending on the type of the fluorochrome. For example *p*-phenylenediamine in buffered glycerin or polyvinyl alcohol mounting medium retards fading of FITC, but not that of TRITC (37). Sodium azide, dithiothreitol, dithioerythritol, sodium hydrosulfite (sodium dithionite), *n*-propyl gallate, ascorbic acid, and 1,4-diazobicyclo(2,2,2)-octane (DABCO) are also useful in retarding photobleaching (Table 3).

1. Fading During Storage

Immunofluorescent-stained preparations do not keep well, as they exhibit higher background fluorescence on storage. In our experience, slides mounted in polyvinyl alcohol can be stored for prolonged periods, when kept at −20°C for up to 1 year or even longer.

F. Types of Fluorescence

The fluorescence observed on examination under the fluorescence microscope can be categorized into the following:

1. Desired or Specific Staining

Specific staining is a true staining as a result of antigen–antibody reactions. The intensity of this reaction is a function of the antigen concentration, the antibody concentration, and the degree of labeling of the antibody to the fluorochrome (fluorescein/protein ratio).

2. Undesired But Specific Staining

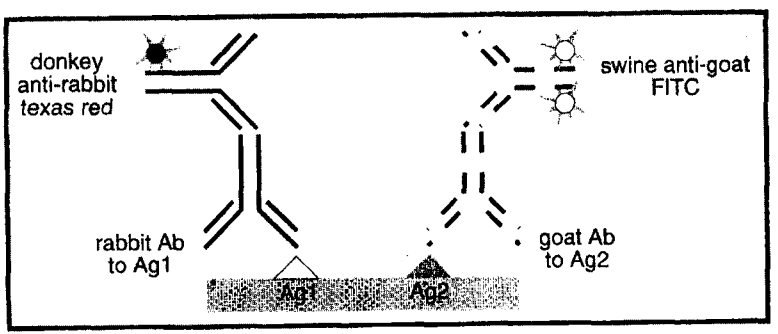
These undesired types of reactions could be due to the presence of cross-reactive antibodies in the conjugate to the tissue substrate other than to the target antigen. This type of staining reaction could be minimized either by the use of affinity-purified conjugates to the target antigen or by absorption of the conjugate with cross-reactive antigens.

3. Autofluorescence

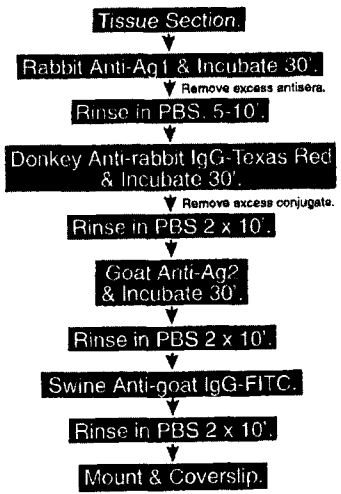
Autofluorescence is due to the presence of natural substances in the specimen that fluoresce on excitation with the UV light. Sometimes this autofluorescence is a nuisance and may conceal or be confused with the specific fluorescence. In tissue sections, collagen and elastic fibers quite often exhibit autofluorescence. In such cases, an examination of the sections without treatment with the conjugate may help in distinguishing autofluorescence from other types of fluorescence.

Table 3 Chemicals Effective in Photobleaching of Immunofluorescent Reactions

Chemical	Recommended concentration (g/L)
p-Phenylenediamine	1
Sodium azide	25
Sodium iodide	25
1,4-Diazobicyco-(2,2,2)-octane (DABCO)	33.8
n-Propyl gallate	10
Sodium hydrosulfite (sodium dithionite)	3.5



(a)



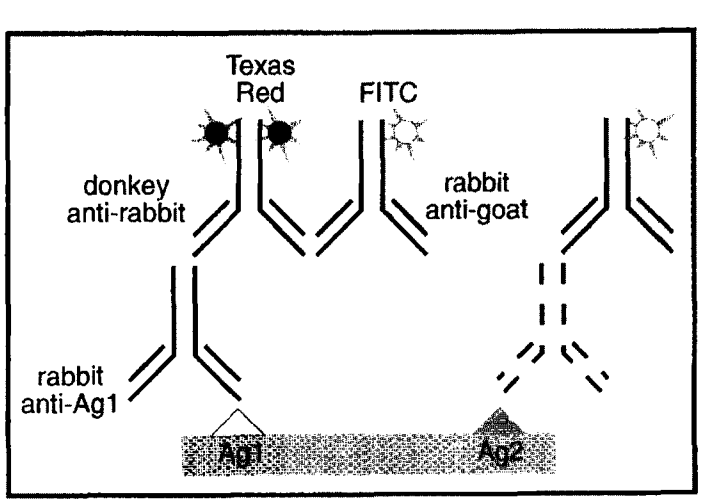


Figure 10 Double indirect immunofluorescence: (a) Standard and (b) modified to display two antigens with one filter.

4. Induced Fluorescence

Induced fluorescence refers to the conversion of a nonfluorescent substance in the tissue into a fluorescent compound by treatment with a nonfluorescent reagent. The most common example is the formaldehyde-induced fluorescence of the tissues.

5. Nonspecific Staining

Nonspecific staining has been attributed to the hydrophobic and ionic interactions of the highly fluorescinated conjugate with the antigenic substrate.

IV. TISSUE FIXATION

To preserve the tissue and cell morphology and to retain the antigenicity of the substrate, it is essential to fix the tissue block, tissue section, cell, or smear preparations. In certain situations, certain fixatives, in addition to preserving the morphology, may either alter the antigenicity, render the cell impermeable to reactions with the antibodies, or enhance the nonspecific background reactions. Thus, one may have to select a fixative that is a compromise between preserving the morphology and maintaining the antigenicity with minimal background reactions. The selection of the fixative may vary according to the technique employed. For example, the choice of the fixative may be different for immunofluorescence, as compared with enzyme immunohistochemical staining. The various fixatives that have been employed in immunohistochemistry include the following:

A. Acetone

Acetone is an excellent preservative and is used frequently in immunohistochemistry on tissue sections and cell or tissue imprint preparations. Even though acetone is an excellent fixative on tissue sections and cell smears, because of its limited penetrating ability, it is not used on tissue blocks.

B. Ethanol

Ethanol fixation is useful primarily for antigens that display carbohydrate-containing epitopes, such as for cell surface antigens. Similar to acetone, ethanol is also a poor penetrative fixative and, hence, is not the one of choice for tissue blocks.

The acetone and ethanol fixatives are employed primarily on cryostat sections, and the following procedure is usually employed.

1. Cut 2- to 4-μm–thick sections of a frozen tissue block and place them on glass slides.
2. Air-dry the slides at room temperature for 10–15 min.
3. Fix in acetone or ethanol for 5–10 min and air-dry.
4. Proceed with immunostaining.

C. Formaldehyde

Formaldehyde is the most common fixative for tissues that are going to be embedded in paraffin. Generally, formalin-based (40% formaldehyde in water) fixatives are excellent for enzyme immunohistochemical staining methods. One disadvantage of the formalin fixative is that some of the antigens may not be demonstrable after fixation in formaldehyde. Usually in such cases, proteolytic digestion of the section before immunostaining may be useful. In such situations the following protocol is usually employed.

1. Place paraffin sections on slide.
2. Place slides in an oven set at 60°C for 10–20 min.
3. Transfer the slides to xylene or xylene substitute for 3 min × 2.
4. Transfer the slide to absolute ethanol for 3 min × 2.
5. Transfer to 95% ethanol for 3 min × 2.
6. Rinse in PBS or TBS buffer.
7. Immunostain.

In situations for which there is a need to trypsanize, the sections after the hydration step (step 6), are then treated with 0.1% trypsin solution in this pH 7.6 buffer for 5–10 min at room temperature. The sections are then rinsed in water and are ready for immunostaining.

In addition to the foregoing fixatives, other fixatives, such as peroxidase-lysine-paraformaldehyde (PLP) or Bouin's solution, are also used for tissue fixation.

V. CONJUGATE

To achieve an optimal performance of the immunohistochemical reaction, in addition to proper processing of the specimen, the selection of a conjugate of the desired specificity is important. Table 4 lists various characteristics of the conjugates that affect the performance of the immunohistochemical reactions and the methods of analyzing them.

A. Specificity

Specificity is a measure of the degree of cross-reactivity of the conjugate to dissimilar antigens or epitopes. For commercial conjugates, even though they are usually of good quality and, in many cases, provide the desired specificity, it is the responsibility of the end user to make certain that the reagents obtained possess the desired specificity. The specificity of the conjugates can be determined by simple methods, such as gel diffusion and immunoelectrophoresis (IEP) (14). However, the best method to determine the specificity, should be the method in which these conjugates are going to be used. In my experience, gel diffusion and IEP methods do not afford the desired reliability for evaluating the conjugates that are going to be used in immunohistochemical methods. The best method in such cases would be the immunohistochemical method on a number of tissue substrates with restricted antigen specificity. For example, in evaluating secondary conjugates (goat antihuman IgG), class-restricted antinuclear antibody (ANA)-positive sera could be used for determining the specificity.

B. Sensitivity

The sensitivity of the assay method depends on antibody affinity, antibody titer, and the degree of conjugate labeling. The higher-affinity antibodies impart greater sensitivity to the method; the

Table 4 Characteristics of the Conjugates

Characteristics	Assay method
Specificity	IEP, gel diffusion, immunohistochemical reactions on defined substrates
Sensitivity	Antibody concentration: radial immunodiffusion, gel diffusion (unitage), chessboard titrations

Table 5 Determination of Optimal-Working Dilution of Goat Antihuman IgG Fluorescein-Labeled Conjugates

	Goat antihuman IgG conjugate–FITC						
	Ab conc (μg/ml)	100	50	25	12.5	6.25	3.1
	Units	1/2	1/4	1/8	1/16	1/32	1/64
	Dilution	1:16	1:32	1:64	1:128	1:256	1:512
Direct method	Specific staining	+++	+++	+++	+++	++	+
	Nonspecific staining	+/w	–	–	–	–	–
		Use dilution 1:64					
Indirect method	PBS	–	–	–	–	–	–
	NHS 1:10	+/w	–	–	–	–	–
	ANA Pos serum						
	1:80	++	++	++	++	+	+
	1:160	++	++	++	++	+	w
	1:320	+	+	+	+	w	–
	1:640	+	+	+	w	–	–
	1:1280	w	w	w	–	–	–
	1:2560	–	–	–	–	–	–
		Use dilution 1:32					

higher the affinity of the antibody, the lower is the concentration needed of the antigen (analyte) to which the antibody is directed to reach equilibrium; hence, a higher sensitivity.

In addition to the antibody affinity, the antibody titers are also of importance in determining the quality of the immunohistochemical staining. Because the antibody titer denotes the concentration of the antibody, the higher the antibody titer, the higher is the dilution of the conjugate that could be used to obtain optimal specific reaction, with the least degree of nonspecific background staining.

C. Use Dilution

The use of the conjugates at the optimal-working dilution enhances the quality of staining. Optimal-working dilution of the conjugate could be determined by a two-dimensional chessboard titration. The optimal-working dilution can be determined by making a series of twofold dilutions of the conjugate and testing them in a direct method against the antigenic substrate, and in an indirect method against serial dilutions of the serum (Table 5). Although chessboard titrations are of critical importance, the antibody concentration that is usually provided by the manufacturer and the unitage assay, which can easily be obtained, provide a good guide in selecting an appropriate dilution of the conjugate (2,39). As an example, for the various immunofluorescence conjugates, we have determined that, frequently, optimal dilutions of the conjugate contain 25–100 μg/ml of the antibody, which is roughly ⅛–½ antibody units/ml.

VI. CONTROLS

Controls are necessary to ascertain the performance of the immunohistochemical reactions. Without the use of proper controls, the validity of the staining reactions, especially when the reactions are negative, will be subject to questions, and doubts will remain concerning the proper

functioning of the system. The controls that are required in the immunohistochemical reaction test system include those for serum and tissue.

A. Serum Controls

To ascertain whether the staining protocols were properly adhered to and whether the test system was functioning properly serum controls, also referred to as procedural controls, must be included routinely in the working protocol. In our two-step indirect immunofluorescence test methods, we routinely include a positive and a negative serum control. The positive control could be used at a predetermined, fixed dilution when performing a qualitative test. However, we recommend titration of the positive control in an assay system when semiquantitative endpoint titrations are made.

B. Tissue Control

Negative and positive tissue controls can be considered in evaluating the performance of the immunohistochemical reactions. These are of importance, especially in virological studies during which the positive viral reactions need to be differentiated from the negative reactions. However, for studies to detect autoantibodies and for direct immunofluorescence studies, we routinely use a positive tissue control. In such studies, an appropriate negative control is difficult to find and, also, there may not be a need for such a control.

VII. SUMMARY

Immunohistochemistry is a very versatile immunopathological tool for the study of distribution and differentiation of antigens and of the presence of in vivo-bound immune complexes. In addition, these methods are invaluable for detection of circulating antibodies to the various antigens. Such methods may be the only ones of choice in certain situations. For the detection and quantitation of these antigens, it is very essential that the immunohistochemical methods for detecting them are properly standardized, with the inclusion of appropriate controls.

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