Chapter 35

Double Indirect-Immunofluorescent Labeling of Cultured Cells

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1. Introduction

Immunofluorescence is a powerful technique for identifying and localizing intra- and extra-cellular components both in histological sections and in cultured cells of plant or animal origin. Briefly, an antibody, raised against a specific component, is used as a label to map the distribution of the component in the specimen, and then visualized under the light microscope using a fluorescent dye (a "fluorochrome") such as rhodamine or fluorescein. These dyes are excited to fluoresce by microscope illumination of the appropriate wavelength. By using fluorochromes that differ both in the wavelength required for excitation and in the color of light emitted, several components can be mapped within the same specimen.

In this way it is possible, for example, to distinguish cell types within tissues, to identify components involved in cell motility, adhesion and cell–cell recognition or, using monoclonal antibodies, to detect small variations in antigen structure. Specimens must first be fixed to preserve structure and to immobilize components that would otherwise be cross-linked and aggregated by the antibodies used to label them. Cell mem-

branes are then permeabilized by detergent extraction. This allows antibodies to reach and label components within the cell. These procedures can be carried out with minimal damage to cellular structure, so that the distribution of an antigen labeled by immunofluorescence can be related to information given by other optical techniques, such as phase contrast and interference reflection microscopy (*1,2*). Furthermore, by fixing a series of replicate cultures at intervals during an experiment, active cellular processes can be observed, the components involved being recognized by their molecular structure (*1,3*). Some of the different methods available for immunofluorescent labeling are briefly compared below.

In direct immunofluorescence, a fluorochrome is linked to the primary antibody. This fluorescently conjugated antibody binds directly to antigenic sites in the fixed and permeabilized specimen. Direct immunofluorescence is the quickest, simplest method of immuno-labeling, but gives rather weak specific labeling and a relatively high background from nonspecific binding.

In indirect immunofluorescence, the fluorochrome is linked instead to a secondary antibody directed against the primary antibody. Several secondary antibody molecules bind to each primary antibody molecule, thus enhancing the brightness of specific labeling and reducing the relative contribution of background labeling. To distinguish several components in the same specimen, primary antibodies must be raised in separate species, and secondary antibodies must be strictly species-specific and conjugated to different fluorochromes whose excitation and emission spectra overlap to a minimal extent (*see* Table 1).

Protein A (*4,5*) is a cell wall protein (mol wt 42,000) of *Staphylococcus aureus* and binds with high affinity the Fc regions (*see* Note 8) of immunoglobulin (Ig) molecules, especially of the class IgG. Fluorescently conjugated protein A can be used instead of a secondary antibody to cut down background labeling, and is especially useful where background labeling results from the binding of secondary antibodies to cell surface Fc receptors (found on granulocytes, B-lymphocytes, and macrophages) in the specimen. However, only about twofold amplification is obtained in this way, compared with the 7- to 8-fold amplification obtained using a secondary antibody. Moreover, the usefulness of protein A is limited by its lower affinity for immunoglobulins of some species, particularly sheep, goat, and rat, and for some subclasses of IgG in other species (*4,6*).

Biotin-avidin and biotin-streptavidin systems (*5,7*)—biotin, a small vitamin (mol wt 224) that can be linked to antibodies with minimal effect

Table 1
Excitation and Emission Maxima of Common Fluorochromes

Fluorochrome	Abs. max. (nm)	Emiss. max. (nm)	Color of observed fluorescence
Texas red	596	615	Deep red
Lissamine-rhodamine-B	570	590	Red
Rhodamine isothiocyanate (RITC)	554	573	Red
Fluorescein isothiocyanate (FITC)	492	515	Green
Aminomethyl coumarin acetic acid	350	450	Blue

on their biological activity. Egg-white avidin and streptavidin (from cultures of *Streptomyces avidinii*) are tetrameric proteins, both able to bind four molecules of biotin. Streptavidin (mol wt 60,000) binds biotin with an affinity 6–10 orders of magnitude greater than that of an antigen–antibody interaction, and with less nonspecific binding than avidin. Using a biotinylated primary (or secondary) antibody and a fluorescently labeled streptavidin, three (or four) amplification steps are achieved: each antigen molecule binds several antibodies; each antibody has several biotins attached, each of which can bind one streptavidin molecule; and each streptavidin can have several fluorochromes attached. Alternatively, a bridging streptavidin (7) can be used to achieve a further threefold amplification: when bound by a biotinylated primary (or secondary) antibody, the bridging steptavidin retains three free sites able to bind fluorochrome-conjugated biotin molecules.

Whichever of these methods is used, the quality of immunofluorescent labeling depends ultimately on that of the antibodies. Polyclonal antisera generally give a high density of labeling because they contain different antibodies able to bind several sites (determinants) on each antigen molecule. Affinity purification removes most of those that bind to molecules other than the intended target. Monoclonal antibodies have the advantage of specificity for a single antigenic determinant and therefore generally cross-react less with other molecules. Another critical factor is the affinity of the antibody for its antigen. This varies greatly according to the species and method of preparation of specimen material, so that quantitative comparisons can be misleading. Optimal conditions and antibody dilutions for immunofluorescence must be found, with a certain amount of trial and error, for each new specimen and antibody, and those given here are intended as a guide, not as a set of hard-and-fast rules.

2. Materials

1. Cells cultured on coverslips. Quantities used in this procedure are suitable for 15-mm diameter coverslips (circular coverslips are easiest to handle, and most microscope objectives are optically corrected for coverslip thickness no. 1.5).
2. A coverslip rack is useful to ensure equivalent processing of numerous replicate cultures.
3. PBS (phosphate-buffered saline): 0.14*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.
4. PBSB: PBS containing 0.05% bovine serum albumin (BSA). Protein is added to all antibody solutions to compete with nonspecifically binding antibodies. Fetal calf serum (10% w/v) may be used instead of BSA.
5. Fixation buffer: 0.25% (v/v) glutaraldehyde in PBS.
6. Extraction buffer: 0.1% (v/v) Triton X-100 in PBS.
7. Sodium azide: 0.01% in PBS. (EXTREMELY TOXIC. Handle with great care.)
8. Sodium borohydride: 0.05% in distilled water. (TOXIC and liberates hydrogen on contact with water.) Store sodium borohydride dessicated, open as briefly and infrequently as possible, and only dilute immediately before use. Add a small spatula-full to 15 mL of ice-cold water and shake in a sealed container.
9. Clean microscope slides.
10. A water-miscible mountant, such as "Uvinert" (Gurr) or "Citifluor" (Goodwin & Davidson, Department of Chemistry, The City University, London). These contain "anti-fade"ingredients that retard the fading of fluorochromes in light of their excitation wavelength.
11. Primary antibodies (either antisera or monoclonal antibodies purified from ascites fluid) raised against each antigen to be labeled. Try to obtain antibodies raised against material of the same species as your specimen. Antibodies raised against an avian protein may, for example, have very weak affinity for the mammalian equivalent. Use of affinity-purified antisera can eliminate labeling of cross-reacting antigenic determinants. However, even monoclonal antibodies can cross-react with similar determinants on completely different molecules. Cross-reactivity of antibodies can be checked on immunoblots of specimen material.
12. Preimmune sera obtained from the same host species (preferably from the same animal) as each primary antibody. On control specimens, to

test for nonspecifically binding antibodies, these are substituted for the corresponding primary antibodies.

13. Fluorescently conjugated secondary antibodies raised against immunoglobulins of the same species as each primary antibody. If more than one component is to be labeled, secondary antibodies must be sufficiently species-specific to distinguish totally between primary antibodies. To test for cross-reactivity between secondary antibodies, each should be used, on separate control specimens, in combination with each primary antibody. The use of secondary antibodies preadsorbed against sera of other species can obviate the need for this control. Secondary antibodies must be conjugated with fluorochromes separated as far as possible in excitation and emission spectra (*see* Table 1). In combination with fluorescein, Texas red is better than rhodamine in this respect. The blue dye coumarin, recently applied in double-label immunofluorescence, additionally eliminates the excitation of one fluorochrome by fluorescence emitted by the other (*8*).

3. Methods

3.1. Double Indirect-Immunofluorescent Labeling of Cultured Cells (See Note 1)

3.1.1. Dilution of Antibodies

1. Antisera vary greatly in antibody titer and the ideal dilution to use varies also with antibody affinity, specimen thickness, and so on. In a preliminary trial, test on replicate specimens a few dilutions between, 10 and 100 µg/mL. Store all antibodies frozen or lyophilized, and dilute only just before use. Solutions for double-labeling of tubulin and vimentin are described in Table 2. These are given merely as examples and are not intended for use as general recipes.
2. Centrifuge diluted antibodies 30 min at 10,000 x *g*, to pellet any precipitates. Keep antibody solutions on ice, and take care not to disturb the pellet.

3.1.2. Fixation and Permeabilization of Cells

Volumes of solutions are not critical. Use enough to immerse coverslips and avoid their drying out. About 1 mL/coverslip should suffice (*see* Note 2).

1. Rinse coverslips briefly in PBS, then pre-fix 5 min at 37°C in fixation buffer.

Table 2
Suggested Dilutions of Antisera for Double Indirect Immunofluorescent Labeling of Tubulin and Vimentin in Cultured Cells

Primary antibodies	Volume	Final dilution
Rabbit antitubulin antiserum	10 µl	1/25
Goat antivimentin antiserum	5 µl	1/50
PBSB	235 µl	
Preimmune sera		
Rabbit preimmune serum	10 µl	1/25
Goat preimmune serum	5 µl	1/50
PBSB	235 µl	
Secondary antibodies		
FITC-mouse IgG antirabbit IgG	6 µl	1/40
RITC-rat IgG antigoat IgG	4 µl	1/60
PBSB	230 µl	

2. Rinse briefly in PBS, and then permeabilize for 15 min in extraction buffer. Steps 2–4 may be performed at room temperature.
3. Rinse briefly in PBS, and then fix 5 min in fixation buffer.
4. Dip briefly in PBS, and then rinse in three changes of PBS over 30 min.
5. Reduce any remaining aldehyde as follows: Immerse coverslips in FRESHLY MADE UP, ice-cold sodium borohydride solution. Incubate 5 min on ice, shaking dishes continuously. Bubbles should form if the solution is fresh. Drain dishes, add fresh borohydride solution, and repeat.
6. Rinse as in Step 4. Continue rinsing until autofluorescence (resulting from residual aldehyde) is completely quenched (check with a fluorescence microscope).

3.1.3. Antibody Labeling

1. Drain coverslips thoroughly and place, close together but not touching, in lidded Petri dishes lined with moist filter paper (filter paper may be omitted if a humidified incubator is to be used for incubations).
2. Add to each coverslip 60 µL of primary antibody solution (preimmune sera to controls) and incubate 30 min at 37°C (or 1 h at room temperature).
3. Drain excess antibody solution from coverslips (touch edge to filter paper), dip briefly in PBS, and then rinse 45 min to 1 h, using at least three changes of PBS and agitating occasionally. This rinsing must be

very thorough. Because IgG antibodies are divalent, secondary antibodies can cross-link any primary antibodies remaining in solution, forming immunoprecipitates, which will appear as spurious fluorescent structures.

4. Repeat Steps 1–3, using secondary antibodies.
5. Continue rinsing in distilled water, checking occasionally under the microscope for removal of background fluorescence. Use 0.01% sodium azide for the final rinses, to prevent bacterial growth after mounting.
6. Invert each coverslip onto a small drop of water-miscible mountant on a clean glass slide.
7. Observe, using a microscope equipped with epifluorescence illumination and "fluor" type objectives (*see* Notes 3 and 4). Only one fluorochrome may be visualized at a time, although photomicrographs of structures labeled with different fluorochromes may be superimposed on color film (*see* Note 5). The corresponding beam-splitter/interference-filter combination is inserted (and must be correctly oriented) into the microscope body, just behind the objective. This transmits light of the excitation wavelength to the specimen and light of the emitted wavelength to the microscope eyepiece or camera.

4. Notes

1. The procedure given is for cultured cells, but very similar methods are applicable to sections (1–10 μm thick) of frozen or wax-embedded tissue (*8*,*9*). The use of frozen-sectioned material avoids some of the loss of antigenicity incurred by fixation.
2. Rigorous cleanliness is important to avoid contamination with cross-reacting antigens. Use acid-washed glass culture substrata (plastics are easily scratched, trapping reagents, and may themselves be fluorescent), separate, disposable dishes for all incubations and rinses, and separate, clean instruments to avoid cross-contamination. Wear gloves at all times. The amplification inherent in most methods makes early contamination more serious, but dust or air-bubbles included at any stage, and in the optical system, can cause reflections with ruinous effect on image contrast.
3. If labeling is very faint, first check that the fault is not in the optical system. Second, try repeating the method using a higher concentration of the primary antibody. Third, raise the concentration of the secondary antibody. If all else fails, try a third incubation with a tertiary

antibody directed against the secondary antibody and conjugated with the same fluorochrome. The resulting chain of three antibodies should be stabilized, before mounting, by postfixation: Swirl for 15 s in 5% acetic acid/80% ethanol, and then briefly in (large volumes of) 70% ethanol, 50% ethanol and water.

4. High background labeling may be the result of insufficient rinsing, specimen contamination, cross-reactivity or nonspecific binding of antibodies, or antibody binding to cell surface Fc receptors. Figure 1 shows the effect of these factors on the fluorescence image. Unwanted labeling of Fc receptors can be diminished by using a biotin-avidin system, or by replacing the secondary antibody with fluorescently labeled protein A. Alternatively, all antibodies can be replaced with corresponding Fab or $F(ab')_2$ fragments (*see* Note 8). However, some of the antigenicity of the primary antibody resides in the Fc region, and fluorescently conjugated secondary antibodies (or their Fab fragments) raised against Fab or $F(ab')_2$ fragments are rarely commmercially available.
5. Fluorochromes, especially fluorescein, gradually fade when exposed to light of the excitation wavelength, or even to daylight or bright artifical light. Keep specimens in the dark, e.g., foil-wrapped, and avoid illuminating for longer than is necessary in order to focus before taking micrographs. Successive exposures of the same area will need increasing exposure times. If using several fluorochromes, don't panic: illumination of the excitation wavelength for one fluorochrome should not cause fading of another. Fading can be retarded by "antifade" mountants although these tend to have high refractive indices, marring the image obtained with phase-contrast optics. Some watermiscible mountants also allow bacterial growth, which may in time destroy the specimen. Avoid this tragedy by using 0.01% sodium azide for the final rinses before mounting.
6. Poor fixation can impair both the antigenicity of cellular components and the structural integrity of the specimen, and several buffering systems have been developed specifically to preserve cytoskeletal structure during fixation (*10*). Glutaraldehyde is a good fixative for preserving cellular structure. Alternatives are paraformaldehyde (2,3), ice-cold absolute methanol and/or acetone (3), and ethylene glycolyl bis (succinamidyl succinate) (EGS). EGS preserves antigenicity better than either glutaraldehyde or paraformaldehyde, because it forms more widely spaced cross-links in the specimen material. For this

reason, fixed specimens remain rather fragile. Dissolve EGS initially in dimethyl sulfoxide and dilute to 5–10 m*M* in warm PBS immediately before use: it tends to precipitate once diluted if allowed to cool below 37°C. Fixation with methanol or acetone obviate the need for a separate permeabilizing step. Another alternative is to include detergent in the fixation buffer, although this accelerates fixation, making timing and temperature more critical. Try 0.05% Triton X-100, 0.5% glutaraldehyde in the buffer described by Small (*10*). Fix for 4 min at 37°C.

7. Permeabilization extracts a significant amount of cell-surface protein, indirectly disrupting cellular structure. Cell-surface determinants, such as growth factor, hormone, or neurotransmitter receptors and cell adhesion molecules are best labeled on cells that have not been permeabilized. Using permeabilized and unpermeabilized replicates, they can be distinguished from receptors that the cell has internalized.
8. Digestion of the immunoglobulin molecule with papain gives rise to fragments of two types: Fc (fragment crystallizable), which contains domains able to bind staphylococcal protein A and cell-surface Fc receptors, but has no antigen-binding specificity; and Fab (fragment antigen-binding), which is a monovalent antigen-binding fragment, consisting of just one of the two antigen-binding arms of the immunoglobulin molecule. $F(ab')_2$ is a divalent antigen-binding fragment prepared by digestion of immunoglobulin with pepsin.

4.1. Variations of the Technique.

Immunofluorescence has many applications beyond the mapping of a few antigenic determinants within fixed cells and tissues. Modified methods can be applied to living cells (*11,12*) or permeabilized but unfixed "cell models" (*13*), giving a more dynamic picture of cellular processes. Antibodies can also be used as tools to manipulate cellular structures, e.g., to cross-link cell-surface receptors (*14*) or to immunoprecipitate intracellular elements (*15*), and thus investigate the connections and functional relationships between cellular components. Unless (monovalent) Fab fragments are used, the (divalent) primary antibody cross-links cell surface components to form a "patch." The movement of patched determinants in the cell surface membrane can be followed by subsequent fixation and labeling with a secondary antibody (*14*) or by image-intensified video microscopy (*11*), and is of interest in understanding the role of the cell membrane in cell motility.

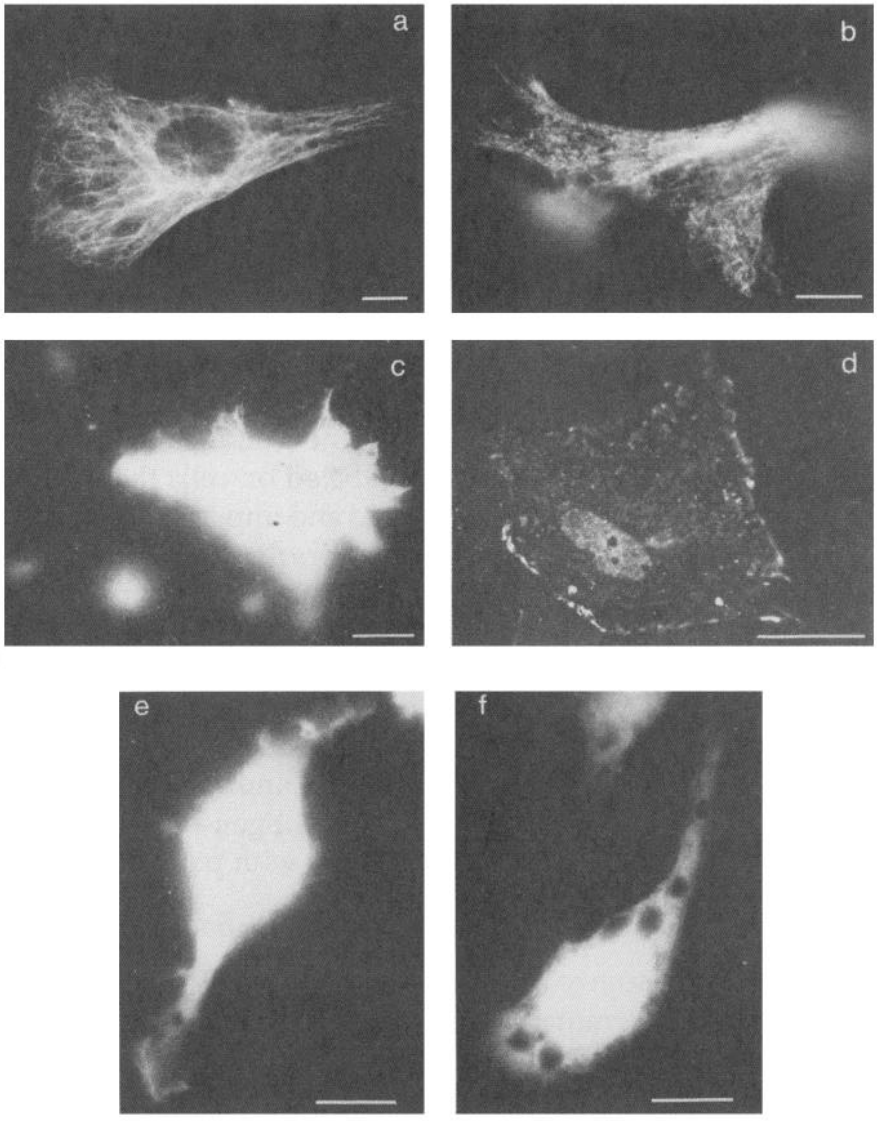

Fig. 1. (a) Chick heart fibroblast labeled with antibodies to tubulin. Cells were fixed 4 min in 0.05% Triton X-100, 0.5% glutaraldehyde in the buffer described by Small (1981) (*10*). (b) Chick heart fibroblast badly fixed with cold methanol and labeled with antibodies to tubulin. Poor fixation is indicated by disintegration of microtubules, and distortion of cellular structure was apparent by phase-contrast microscopy. (c) Chick heart fibroblast labeled with antibodies to tubulin. Incomplete removal of unbound primary antibody has allowed the formation of fluorescent immunoprecipitates, obscuring detail of the cell. **Note:** Incomplete removal of unbound secondary antibody would instead have caused overall brightness of cells and background. (d) Chick heart fibroblast labeled with antibodies to vinculin, a component of cell-substratum adhesions. Not only the marginal adhesions of this cell are labeled but also its nucleus, indicating cross-reactivity with components other than vinculin. Indeed, this antiserum cross-reacted with several nuclear

The links between cytoskeletal components and the ventral membrane of the cell are particularly important in the study of cell adhesion and motility. In immunofluorescently labeled preparations, detail is often obscured by out-of-focus images from other planes within the cell. This problem has been overcome first by studies of isolated ventral membranes of adhering cells (*16*), and second by an ingenious optical technique giving a three-dimensional view of intact, fluorescently labeled cells (*17*).

Further variations of the technique have been used to throw light on the mechanism of cell adhesion in motile and phagocytic processes. Cells are plated onto surfaces derivatized with a "carpet" of a primary antibody (*18*), or of a protein that can later be immunofluorescently labeled (*19*). The resulting pattern of dark patches reveals the regions where tight adhesion of the cell restricts access of the secondary antibody, or where the cell has removed the protein carpet.

The spatial relationships between cytoskeletal and extracellular components have been investigated in ever-increasing detail by the more recently developed technique of immuno-electron microscopy (*20,21* and Chapter 38, this vol). This differs from immunofluorescence in that electron-dense markers such as colloidal gold or ferritin replace fluorochromes, allowing cellular components to be mapped at the much higher resolution of the electron microscope (*21*). Because of the different artifacts produced by preparation for different types of microscopy, immunofluorescence and immuno-electron microscopy are especially valuable in combination (*20*).

Other techniques closely related to immunofluorescence include the labeling of cellular components with fluorescent cytochemical markers, such as the phallotoxins (*3,22*) and heavy meromyosin (*14*), or their substi-

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proteins on immunoblots of chick heart material. (e) Mouse macrophage labeled with antibodies to the membrane protein, talin, associated with cell-substratum adhesions. The antiserum was raised against chick gizzard talin and showed low affinity for mammalian talin. Cell-substratum adhesions are not notice-ably labeled. Instead, the whole cell is labeled by antibody binding to cell surface Fc receptors. Note especially bright labeling of membranous folds–"ruffles"–at both ends of the cell. (f) Mouse macrophage labeled with antibodies to the colony-stimulating factor, CSF-1. Although Fab fragments of the primary antibody were used to avoid labeling cell-surface Fc receptors, specific labeling of cell-associated CSF-1 is obscured by a general cytoplasmic fluorescence because of incomplete quenching of residual glutaraldehyde used in fixation. Exclusion of this fluorescence from intra-cellular vesicles indicates that, unlike that in (e), it is not confined to the cell surface.

All scale bars 10 μm.

tution with fluorescent analogues, which may be incorporated into living cells directly (*12*) or by microinjection (*23,24*). These techniques again are most useful in combination with immunofluorescence. The high contrast of the fluorescence image makes it suitable for video image analysis, and the clear distinction between labeled and unlabeled cells is useful in systems for cell-counting (flow cytometry) and fluorescence-activated cell-sorting (FACS) (*see* Chapters 40 and 41, this vol.).

Acknowledgment

This work was supported by the Cancer Research Campaign.

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