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## CHAPTER 18

# Indirect Immunofluorescence Microscopy in Cultured Cells

**Sally P. Wheatley and Yu-li Wang**

Cell Biology Group  
Worcester Foundation for Biomedical Research  
Shrewsbury, Massachusetts 01545

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The technique of fluorescence immunolocalization has evolved steadily since its first application in the mid-1960s, incorporating innovations in probe chemistry, microscopy, and image detection. This chapter provides an overview of the current status of indirect immunofluorescence for those starting to use the method. It includes both general considerations from cell culture to image detection and several protocols that should serve as an entry point for this technique.

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## I. Introduction

Immunolocalization of antigens within cells and tissues has been a routine procedure in cell biology (see Osborn and Weber, 1982; Wang *et al.*, 1982). Although methods for preserving and probing cells have not altered dramatically, the sensitivity and versatility of this technique have improved significantly over the last decade due to major advances in signal detection, including the commercialization of cooled-charge couple device cameras (CCD), developments in image analysis, and the availability of a new generation of fluorophores. This chapter is offered as an introduction to the technique, particularly indirect immunofluorescence microscopy, and is intended to draw the readers' attention to ways in which its utility can be maximized. The methods illustrated in this chapter use microtubule organization in cultured cells as an example. However, the procedures described are applicable to all immunolocalization studies.

Immunofluorescence localization can be direct or indirect, the principles are the same. In the direct approach, antibodies are linked to a fluorescent probe and used directly to localize the antigen in the sample of interest. In the indirect method, the cell is probed with an unlabeled primary antibody and its location is in turn reported by a fluorophore-conjugated secondary antibody that recognizes the primary antibody. When working with fixed cells, the indirect method is most commonly used, as the primary antibody serves to amplify the abundance of the antigen, enhancing the intensity of the final signal. This method is also more versatile, allowing a range of fluorophores to be used in combination with a single primary antibody.

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## II. Cell Culture

The type of cell that one is working with is often predetermined, e.g., COS cells are commonly used for transfections and HeLa cells for study of transformed cells. However, the optical qualities of the cell can be equally important for obtaining optimal results. The authors have worked extensively with two cell lines: normal rat kidney epithelial cells (NRK) to monitor cell division and intracellular organelle dynamics and Swiss 3T3 fibroblasts to study cell locomotion. Both types of cells grow and spread well on glass. NRK cells, which are

similar to PtK cells have the advantage that they remain spread during cell division (when most other cells round up), a feature that permits high-resolution observations of mitosis and cytokinesis. Without such advantage, a confocal microscope or computer imaging restoration is required to obtain images of comparable resolution.

Cells are cultured directly on rectangular glass coverslips (45 × 50 mm; size 1 or 2 thickness; Fisher Scientific, Pittsburgh, PA), which are passed through a blue flame immediately before plating the cells. These methods of cell culture have been described previously (McKenna and Wang, 1989). Briefly, a sterile coverslip is adhered by vacuum grease to an acrylic block with a circular aperture (35 mm in diameter) to create a chamber. Cells are passaged directly into the chamber at the appropriate density 48 hr before experimentation. This arrangement is ideal for viewing and manipulating cells using an inverted microscope. However, as upright microscopes are more commonly used, most laboratories culture cells on smaller coverslips, placed in plastic petri dishes or multiwell plates.

Some cells need encouragement to grow on glass. In these cases, coverslips are incubated for 30 min in a sterile solution of poly-L-lysine (0.1–1 mg/ml) and washed with sterile water before seeding (Mazia *et al.*, 1995). If cells are grown in suspension, e.g., HeLa cells or yeast cultures, they need to be concentrated, then adhered to a poly-L-lysine coated coverslip before observation. Cells can be attached by gravity or with a “cytospin” before or after processing for immunofluorescence (for details, see Harlow and Lane, 1988). The goal is to obtain an even distribution of cells, separated by a workable distance, on the coverslip. For this purpose, immunostaining before mounting may be preferable as a serial dilution of cells can be made onto a number of coverslips.

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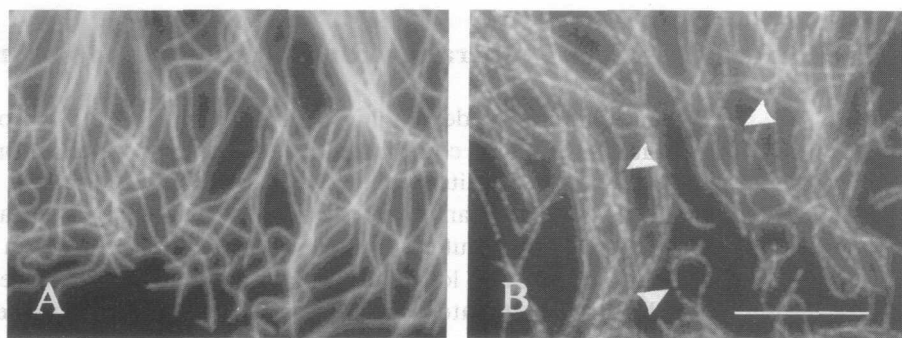
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### III. Sample Preparation for Immunocytochemistry

When choosing or developing an appropriate sample preparation protocol there are three main concerns: (1) that the true distribution of the antigen is preserved, (2) that its antigenicity is not compromised by the fixative, and (3) that the antibody can access the antigen. Ideally, to be certain that the probe reports the true distribution of the antigen, a variety of fixation protocols should be tried and the same localization pattern observed with at least two different procedures. Unfortunately, artifacts introduced from a number of sources can generate disparate images of the same antigen. For example, organic solvents dehydrate the specimen and can cause gross distortions in cell structure; certain buffers, particularly those containing high concentrations of phosphates, can cause precipitation of proteins and cations, depositing them in nonphysiological locations. This may be part of the reason why cytoskeleton buffer (CB) (Small *et al.*, 1982) is better than phosphate-buffered saline (PBS) as the diluent for fixatives and detergents (see Section VII).

Figure 1 demonstrates good versus poor microtubule preservation in two interphase NRK cells. Using glutaraldehyde and mild extraction with Triton X-100, microtubule ultrastructure is preserved well, revealing a network of continuous filaments (Fig. 1A). However, microtubule structure is poorly retained with formaldehyde fixation and they appear as beads along invisible threads (Fig. 1B). All samples are prepared similarly for immunocytochemistry, from yeast to *Drosophila* and tissue culture cells, although special procedures are applied after initial fixation such as removal of the cell wall from yeast (Alfa *et al.*, 1993; Balasubramanian *et al.*, 1997) and the vitelline membrane from *Drosophila* embryos (Theurkauf, 1994) to facilitate the penetration of antibodies. Particularly yolkly specimens such as sea urchin and *Drosophila* embryos may also need to be "cleared" after preparation to minimize internal light scattering and autofluorescence (Wright and Scholey, 1993; Theurkauf, 1994).

The authors routinely use three fixatives: glutaraldehyde, formaldehyde, and methanol. Ethanol and acetone are also commonly used. Glutaraldehyde provides the highest degree of sample preservation by cross-linking the side chains of neighboring proteins, locking them in place with little morphological damage. At the molecular level, however, chemical modifications induced by glutaraldehyde can cause severe alterations in the protein structure that result in loss of antigenicity. Therefore, although glutaraldehyde is preferred for the preservation of ultrastructure, it is not feasible for many antigens. Formaldehyde also cross-links proteins. Although poorly understood, its action is known to be unstable and can be partially reversible in neutral solutions (see Means and Feeney, 1971; Harlow and Lane, 1988).

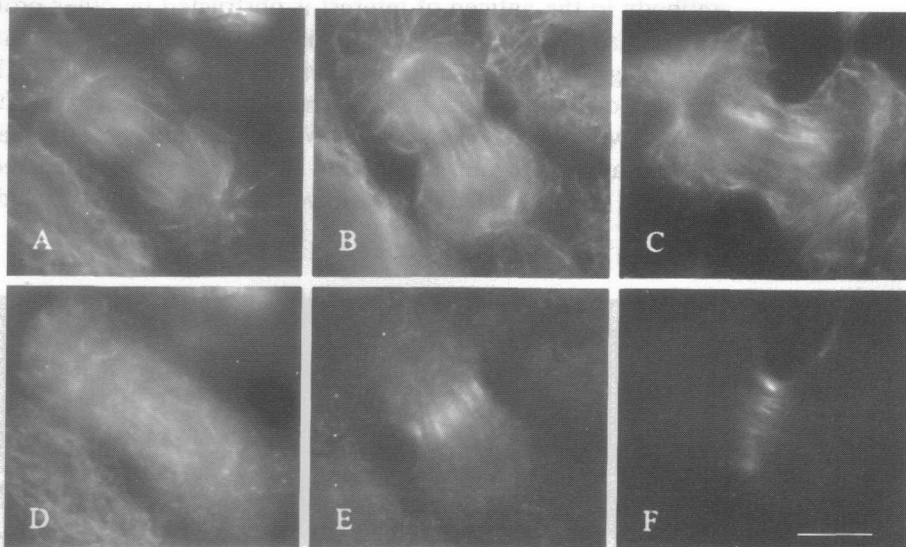


**Fig. 1** Microtubule preservation in the peripheries of two interphase NRK cells fixed with (A) glutaraldehyde/Triton and (B) formaldehyde/Triton, as detailed in Section VII (extraction in each case was for 2 min at room temperature with 0.1% Triton). Both cells were probed with a monoclonal antibody directed against  $\beta$ -tubulin (Amersham; 1/10) and visualized using an FITC-conjugated antimouse whole IgG secondary antibody (Sigma; 1/50). (A) microtubules are revealed as continuous filaments forming a network throughout the cell. (B) Staining is discontinuous (arrowheads), giving the microtubules a broken appearance. Bar: 10  $\mu$ m.

Methanol preserves protein structure by dehydration and coagulation. It extracts lipids simultaneously while fixing, causing nonanchored proteins to leak from the cell. Like other organic solvents, methanol can also cause soluble proteins to become deposited onto remaining cell structures. Because all these properties can generate a false pattern of localization, organic solvents tend to be used when studying antigens that are well anchored.

Knowing certain biochemical properties of the antigen can help in solving the problem when preservation is suboptimal. In many cases, however, the identity or biochemistry of all antigens involved may not be known and refinements and compromises will have to be made over a course of trials. As an example, microtubules are sensitive to cold temperatures and free calcium ions. They retain their antigenicity unusually well in glutaraldehyde prepared in warm cytoskeleton buffer. However, if they are to be colocalized with another protein, it is often necessary to use methanol supplemented with EGTA to optimize the preservation and antigenicity of both components (see Fig. 2).

All fixation protocols run the risk of destroying antigenic sites, and problems can arise from overfixation and underfixation. As there are no hard and fast



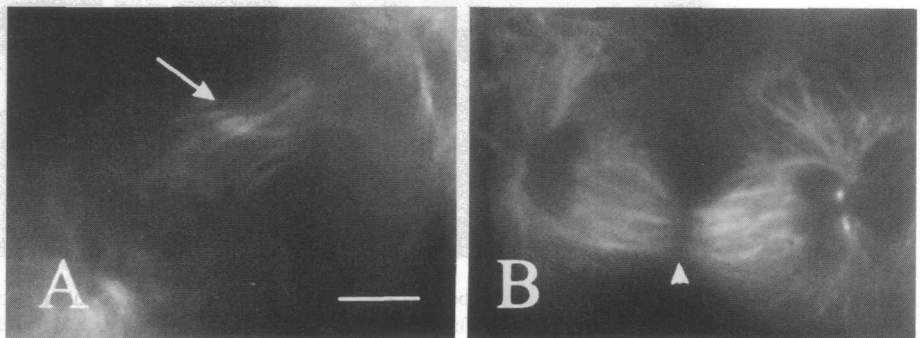
**Fig. 2** Double immunolabeling of  $\beta$ -tubulin (A–C) and the microtubule motor, CHO1 (D–F), in three telophase NRK cells fixed with glutaraldehyde (A, D), formaldehyde (B, E), or methanol (C, F), according to the protocols in Section VII. Using the glutaraldehyde protocol, the microtubule structure is well preserved (A), but CHO1 distribution appears to resemble that of the microtubules (D). With formaldehyde the microtubules appear broken (B), yet CHO1 is located in its true position at the developing midbody (E). Methanol fixation preserves the microtubule structure better than formaldehyde (compare C and B) and reports a similar CHO1 distribution as did formaldehyde fixation (compare F and E). Methanol fixation is therefore chosen in this colocalization study. Bar: 10  $\mu$ m.

rules regarding the reactivity or sensitivity of antigens to different fixatives, most laboratories have a favorite set of basic protocols that can be modified when necessary (see Section VII).

To facilitate antibody penetration, cells are “extracted” or “permeabilized” with nonionic detergents or organic solvents during or after fixation. The most commonly used permeants are nonionic detergents, acetone, and methanol. Detergents can also be added to the initial fixative solution to assist its penetration within the cell. This is particularly helpful when dealing with thick or yolkly specimens such as *Drosophila* or sea urchin embryos (Wright and Scholey, 1993).

If the antigen in question is anchored within the cell, extraction generally does not cause problems, as further extraction may even reduce nonspecific background staining. However, if the antigen is soluble, extraction can remove it completely (see Melan and Sluder, 1992). Extraction with commercially available, nonionic detergents such as Triton X-100, Tween 20, Brij 35 and Nonidet P-40 is more gentle and can be adjusted to give the best results by varying concentration, temperature (although not when fixing and extracting tissue culture cells simultaneously), or duration of incubation.

Another problem encountered is “epitope shielding” where access of the antibody to the antigen of interest is obstructed by other proteins or material. This is classically represented by tubulin immunolocalization at the midbody of a dividing mammalian cell. In a living NRK cell injected with labeled tubulin the midbody appears as the brightest band of fluorescence within the cell (Fig. 3A, arrows; see also Wheatley and Wang, 1996). In contrast, in a fixed cell probed with an antibody against  $\beta$ -tubulin the midbody is manifested as a dark zone (Fig. 3B, arrowhead). Tricks to unveil masked epitopes include treatment with



**Fig. 3** Comparison between midbody appearance in a living NRK cell microinjected with rhodamine-labeled tubulin (A) and a glutaraldehyde-fixed NRK cell probed with anti- $\beta$ -tubulin (B), as described in Fig. 1. Microinjected rhodamine-tubulin incorporates readily into the midbody. Because of the overlap of microtubules in this region, the central part appears most intensely fluorescent (A, arrow). In contrast, in the fixed preparation, antibodies have difficulty accessing midbody microtubules, hence the region appears as a dark zone (B, arrowhead). Bar: 10  $\mu$ m.

protease, trypsin, and inclusion of the denaturant sodium dodecyl sulfate (SDS) in the fixative (see Harlow and Lane, 1988; Ding *et al.*, 1995).

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## IV. Antibodies

This section briefly outlines antibody handling and selection. For a more complete account and for details on antibody structure and production, readers are referred to the laboratory manuals of Harlow and Lane (1988) and Celis (1994).

### A. Handling

Antibodies are robust proteins; however, repeated freeze–thaw cycles should be avoided. Undiluted stocks should be aliquoted into appropriate working volumes and stored at  $-20$  or  $-80^{\circ}\text{C}$ . Secondary antibodies can be maintained undiluted at  $4^{\circ}\text{C}$  for up to 6 months. Those conjugated to a fluorochrome should be stored in a light-tight container. For immunocytochemistry, *all* antibodies should be routinely diluted immediately before use in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. BSA blankets nonspecific epitopes that may interact with the antibody when it is presented. Other commonly used concealants include dry milk powder (up to 10%), fetal calf serum (up to 10%), or higher concentrations of BSA. Azide is included to deter the growth of microorganisms (note that azide is extremely toxic!). Before use the diluted antibody is spun at top speed in a microfuge (at room temperature) for 30 min to remove debris such as unconjugated dye and aggregated antibodies.

### B. Titration

A new primary antibody should always be tested with a known secondary antibody and vice versa. New antibodies should be tested in serial dilution, e.g., 1/10, 1/100, 1/500, and 1/1000, paying close attention to the range suggested by the manufacturer (if applicable). Occasionally, when using ascites fluid, dilution may have to be increased to 1/10,000. However, if monoclonal antibodies are supplied as tissue culture supernatants, they are often used undiluted. When using purified antibodies it is common practice to express the concentration of the antibody in micrograms per ml, particularly if these are noncommercial stocks. For commercially available antibodies it suffices to indicate the dilution factor used for each antibody.

### C. Primary Antibodies

Antibodies recognize and bind to specific “epitopes” or “antigenic determinants.” They can be either monoclonal, recognizing a single epitope, or poly-

clonal, a collective of antibodies recognizing different antigenic determinants of the same molecule. Most commonly, monoclonal antibodies derive from mice and polyclonals from rabbits. The specificity of monoclonal antibodies has obvious advantages. For example, after assembling into microtubules, tubulin subunits undergo a number of posttranslational modifications, including detyrosination. Therefore, ID5, a monoclonal antibody developed against the detyrosinated end domain of  $\alpha$ -tubulin (Wehland and Weber, 1987), can recognize a specific subset of microtubules and give a markedly different localization pattern to that of total  $\beta$ -tubulin (Fig. 4). Although sometimes desirable, this specificity can be confusing with monoclonal antibodies reacting against different parts of the same antigen reporting different distributions (see Oka *et al.*, 1994). As a consequence of their specificity, monoclonal antibodies tend to be more susceptible to preparation artifacts than polyclonal antibodies (see later). Nevertheless, they tend to cause fewer problems associated with nonspecific binding. When combining immunocytology with genetic manipulations, it is important to know which part of the molecule is recognized by the antibody. In such studies, polyclonal antibodies may be preferable as they react with multiple parts of the molecule and are less likely to be completely impeded by alterations in the protein. A compromise between monoclonal and polyclonal antibodies is the use of pooled monoclonal antibodies as these are not limited to one antigenic determinant, yet minimize nonspecific staining.

In addition to purified antibodies, whole immune serum is sometimes used. Rigorous controls should be performed when using sera as additional antibodies are present that can give false staining patterns. The most pertinent control is staining with preimmune serum from the same animal, if possible. If this is unavailable, serum from a control animal can be used as a minimal requirement.

Autoimmune serum from humans has led to the identification of many interesting antigens. Unfortunately, as such serum tends to be obtained from patients with severe illnesses, their supply is highly unpredictable (although this can be circumvented using molecular techniques). Because the mortality of hosts can lead to batch variation, it is wise to reassess new batches of antibodies on arrival both for the appropriate titration and to ensure that the staining pattern has not deviated. Monoclonal antibodies have the distinct advantage that, if hybridoma cell lines are maintained properly, a supply should be available *ad infinitum*.

The quality and specificity of a primary antibody are assessed by standard Western blotting. The antibody should recognize only target antigens. Often it is necessary to perform affinity purification using either chromatographic techniques or on a microscale (Hammerback and Vallee, 1990) to remove nonspecific reactivity. After affinity purification, antibodies should be reexamined by Western blotting. For details on Western blotting and affinity purification, readers are referred to Harlow and Lane (1988).

#### D. Secondary Antibodies

Immunologically, the primary antibody used dictates the secondary antibody required. The secondary must be targeted against the appropriate class of immu-



noglobulin (or light chains) of the species in which the primary was made and must be manufactured in a distinct species. Most secondaries are polyclonal and are generated in a wide range of species. It is necessary to perform controls on unknown secondary antibodies. In particular, incubation with a secondary antibody alone will report nonspecific interactions between cellular components and the secondary antibody or its conjugate. If primary antibodies are applied as antiserum, cells should be tested by incubation with preimmune serum then with secondary antibodies.

### E. Double Labeling

When immunolocalizing more than one protein within a sample, primary antibodies should be from different species and fluorochromes must contrast. There are combinational and sequential options to the application of antibodies when labeling multiple antigens, e.g., for double labeling: (1) primary  $x$  then secondary  $x$  then primary  $y$  then secondary  $y$ ; (2) primary  $x$  then primary  $y$  then secondary  $x$  then secondary  $y$ ; (3) primaries  $x$  and  $y$ , then secondaries  $x$  and  $y$ ; or (4) primary  $x$  then primary  $y$  then secondaries  $x$  and  $y$ . Despite being more laborious, the authors tend to use the first sequence punctuated with extended incubations in blocking solution (PBS/BSA/azide) to minimize cross-reactions between antibodies.

Although up to five different components have been labeled simultaneously within a single cell, rarely is immunostaining performed with more than two probes as, in addition to confusion arising from combining antibodies from different species, the availability of fluorochromes becomes limiting. Although triple labeling can be achieved using immunological probes entirely (Herzog *et al.*, 1994), it is commonly achieved by double immunolabeling and counterstaining once (see Sections VII,F) or by immunolocalizing a single antigen and then counterstaining with two fluorescent probes.

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## V. Signal Detection

### A. Fluorophores and Filter Sets

Fluorophores are molecules that absorb light of one wavelength and emit light at a longer wavelength. They differ in their absorption and emission spectra, extinction coefficient, quantum efficiency, and stability. Most fluorescence microscopes are fitted with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filter sets as these two fluorochromes have been available for many years. However, secondary antibodies conjugated to alternative fluorophores are now available and may be preferable. Useful information regarding fluorophores can be found in relevant manufacturers' catalogs, such as Molecular Probes (Eugene, OR) and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

Three commonly used red fluorophores are TRITC, Texas red, and indocarbocyanine 3 (Cy3). There are advantages and disadvantages to each. As TRITC is the traditional choice, most microscopes have a filter set designed for its maximum absorption and emission. Cy3 has similar excitation and emission spectra (see Table I), but is brighter and more photostable. Therefore, one might select Cy3 over TRITC when an antigen is present in low abundance or the signal with TRITC is weak.

When double labeling a sample, it is common to use FITC and a red fluorophore. Cy3 and FITC or TRITC and FITC, although often used, are less favorable as their excitation peaks are close enough to cause considerable overlap or “bleed through” between channels. These pairs should be used only with carefully tested band-pass filters. A better combination is FITC and Texas red as their spectra are well separated. Using a TRITC filter set, Texas red absorbs and emits light at margins of its spectra but can still be detected (particularly by a CCD camera). To maximize the signal from Texas red, filters that allow its peak excitation and emission light to pass can be obtained. (One caveat with Texas red, however, is its nonspecific affinity for proteins, which may cause high background staining.) An alternative approach is to find a substitute for FITC, such as indodicarbocyanine (Cy2) or a probe of a different wavelength, e.g., aminomethylcoumarin (AMCA or coumarin) or a short wavelength BODIPY.

## B. Green Fluorescent Protein

With the introduction of green fluorescent protein (GFP) as a reporter molecule, it is now possible to visualize directly a fluorescently tagged protein within a living cell using transfection (see Stearns, 1995). Although *in vivo* application is its major strength, GFP also has distinct advantages in fixed preparations (although avoid using methanol as it denatures GFP). First, as GFP is manufactured by the cell itself, the problem of target accessibility is eliminated and extraction becomes unnecessary. Second, GFP can be excited using the regular FITC channel; although this is not its peak absorption, when excited in this way GFP is relatively photostable, making it preferable to FITC as a fluorophore (Stearns, 1995). Third, mutations in GFP are now available that offer a range

**Table I**  
**Absorption and Emission Peaks of**  
**Commonly Used Fluorophores**

Fluorochrome	Absorption (nm)	Emission (nm)
FITC	492	520
Cy3	550	570
TRITC	550	570
Texas red	596	620

of absorption and emission spectra, providing a greater scope in fluorophore selection (Heim and Tsien, 1996).

### C. The Microscope

A number of adjustments can be made to the microscope to maximize observations.

#### 1. Illumination

Fluorescence microscopes are usually fitted with a mercury arc lamp. The intensity of emitted light can be altered by interposing neutral density filters between the light source and the sample; alternatively, the light source can be adjusted through an electronic controller available from Carl Zeiss. Also commonly used are xenon arc lamps, which give a more uniform excitation across the visible and UV light spectrum, and quartz halogen lamps, which are dimmer and more suited to fluorescence imaging in live cells.

#### 2. Objective Lenses

In addition to selecting an appropriate magnification, there are several other considerations in choosing an objective lens for immunofluorescence: degree of correction, material, number of lens elements, and its numerical aperture. Although lens correction is desirable to prevent spherical aberration and color splitting, it requires additional lens elements, which lead to loss of signal. In addition, some glass material is noticeably less autofluorescent than others. The most critical parameter, however, is the numerical aperture (NA). In epifluorescence microscopy, because the objective lens serves as both the condenser and the detector, signal intensity is proportional to the fourth power of the NA.

#### 3. Field Iris

Microscopes are equipped with an adjustable field iris. Although observations are generally made with the field iris fully open, it is often advantageous to close the iris around a particular region to minimize light scattering and remove out-of-focus haze. It can also be used to mask areas of high signal (e.g., densely crowded areas) so that less brightly labeled regions can be viewed.

### D. Cameras

As mentioned in the Section I, major advances have been made in the instrumentation for recording microscope images. Although it has been common practice to use 35-mm cameras fitted with high-sensitivity film (>400 ASA), these are being superseded with cooled charge-coupled device cameras, which can

detect very low levels of light. The high sensitivity of CCDs translates as a decrease in exposure time and also makes dimmer fluorophores a viable option. This decrease can be substantial—an order of magnitude over a regular 35-mm camera. CCD cameras also have the advantage that they are operated via a personal computer, hence images can be viewed, exposure times and image quality optimized, and data filed easily and rapidly. However, without additional coating, CCD chips do not respond well to ultraviolet (UV) light. Even with UV-sensitive coating, CCDs are more sensitive to long wavelength dyes such as Texas red, TRITC, and Cy3 than to Hoechst 33258, DAPI, or fluorescein. On the other hand, our eyes are extremely inefficient in detecting red light so without a CCD camera signals often appear deceptively weak at long wavelengths.

Although color CCD cameras are available, black and white models are more sensitive as signals of virtually all wavelengths are accumulated into one image. When using black and white CCD cameras, color images can be generated through the application of pseudocolor to images of different channels and merging with computer programs such as Adobe Photoshop 4.0 and Metamorph (Universal Imaging, West Chester, PA). Alternatively, the use of 35-mm high-speed color film remains as a simple option for recording color fluorescence images.

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## VI. Miscellaneous Issues

### A. Counterstaining

When presenting immunofluorescence data it is often necessary to familiarize the audience with the general appearance of the cell before indicating the specific points of interest. One commonly encountered problem is that the fixation protocol that preserves the antigen may leave the cell looking ravished in phase contrast or bright field. An alternative way to orientate the reader is to counterstain cells with a fluorescent marker such as Hoechst 33258 or DAPI to show the position of the chromatin or fluorescently tagged phalloidin to localize filamentous actin (see Sections VII,F). In addition, because fungi have the advantage that the cell wall and septa stain with the laundry brightener Calcofluor, dividing cells can be identified easily in fixed preparations (Alfa *et al.*, 1993; Balasubramanian *et al.*, 1997). Counterstaining is a quick, easy, and reliable way of comparing the localization of the antigen with other structures in the cell. However, as with double immunostaining, fluorochrome selection becomes more constrained.

### B. Mounting Cells

When viewing cells with fluorescence they should be mounted in an appropriate medium. This should contain an antibleaching compound such as *n*-propyl gallate or *p*-phenylenediamine, should be viscous, and should be in the pH range 7–8

(FITC prefers pH 8). The authors make their own stock (see Section VII,H), but many mounting media are available commercially. To prevent oxidation the coverslip should be sealed to the slide with nail varnish. If the sample is going to be kept for a long time (months–years), a self-hardening mounting medium such as Gelvatol or Mowiol is preferable (see Harlow and Lane, 1988).

### C. Cell Morphology

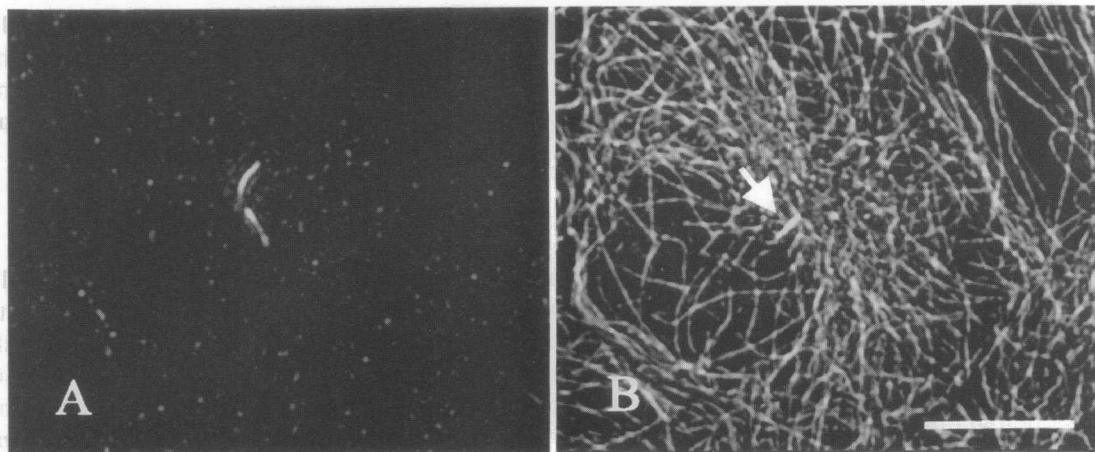
It is important to become acquainted with the morphology of control cells and their substructures before studying experimental cells. It is also important to consider the dynamics of structures within living cells. For example, microtubule organization alters rapidly, particularly during mitosis. Likewise, many motility events, both intracellular and locomotory, take place as cells explore and respond to environmental cues. Thus cells can look considerably different from one moment to the next. Using immunocytochemistry, only static images of the cell are obtained. Nevertheless, some information regarding the dynamic nature of the cell or its constituents can be inferred through cell cycle staging and comparison of structures between individual cells. When dealing with unfamiliar structures, it may be advantageous to take snap shots at low magnification first, as these can be used later for unbiased statistical analyses.

### D. Focal Plane

Structures of interest are often found at different focal planes. For example, in kidney cells the primary cilium usually sits above the interphase nucleus, protruding into the external medium (Wheatley *et al.*, 1994). Unless one focuses above the cell, the primary cilium is undetectable (see Fig. 4). Focusing through the specimen is a good twitch to develop, but better still is the use of an optical sectioning device, which operates by the same principle as in a confocal microscope, but is a simple attachment to the conventional light microscope (Wang, 1997).

## ==== VII. Laboratory Protocols for Fixation/Extraction

The basic protocols are listed from the quickest (methanol) to the most involved (glutaraldehyde). Empty coverslip boxes are ideal as “fixation containers.” When using smaller coverslips, disposable multiwell plates or petri dishes may be more appropriate. Prevent coverslips from colliding, and be aware which side has cells attached: the cell side will appear matt rather than glossy. If preparing cells in suspension, it is important to ensure that they are exposed uniformly to solutions. This is best achieved by performing incubations in microfuge tubes fitted to a cell rotator. It is often convenient to place these tubes in empty black 35-mm film canisters to contain any leaks and to protect the sample



**Fig. 4** Deconvolved and reconstructed images (90° perspective) comparing the distribution of detyrosinated  $\alpha$ -tubulin (A) and total  $\beta$ -tubulin (B) in two interphase NRK cells. (A) The cell was fixed using the formaldehyde protocol (Section VII,B), probed with undiluted monoclonal antibody (ID5; a tissue culture supernatant), and visualized using FITC-conjugated antimouse IgG (Sigma; 1/50). Detyrosinated  $\alpha$ -tubulin specifically labels the primary cilia that project from the top surface of the cell. (B) The cell was fixed using the glutaraldehyde method and probed with anti- $\beta$ -tubulin as detailed in Fig. 1. The entire interphase microtubule network can be seen, including a primary cilium (arrow) Bar: 10  $\mu$ m.

from light where necessary. When using chilled organic solvents, place coverslip sideways into a 100-ml glass beaker. The large volume and small surface area help prevent heat exchange. Fixatives should be made up in disposable containers or in bottles designated for fixatives only. Buffer pH is adjusted at ambient temperature (22°C); the solution is then warmed or chilled as appropriate. Buffers are stored at 4°C. Other reagents are stored as detailed below. Unless stated, reagents are supplied by Sigma Chemical Co.

### Buffers

1. Phosphate-buffered saline, pH 7.4.
2. Cytoskeleton buffer
  - a. 1 $\times$  CB: 137 mM NaCl, 5 mM KCl, 1.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 2 mM EGTA, 5 mM PIPES, and 5.5 mM glucose, pH 6.1.
  - b. 1.3 $\times$  CB: 182 mM NaCl, 6.6 mM KCl, 1.46 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM MgCl<sub>2</sub>, 2.7 mM EGTA, 6.6 mM PIPES, and 7.3 mM glucose, pH 6.1.
3. EGTA: Store stock solution (100 mM, pH 6.0) in a plastic, not glass container.

### *Fixatives and Permeabilizing Reagents*

1. Acetone: store at room temperature.
2. Formaldehyde: 16% stock (methanol free), supplied in 10-ml ampoules (Electron Microscopy Sciences, Fort Washington, PA) and stored at room temperature. (Ampoules should be discarded if opened for more than 1 week. Avoid prepared solutions of formaldehyde that contain methanol such as formalin. Some researchers prefer to make a fresh solution of formaldehyde from paraformaldehyde powder.)
3. Glutaraldehyde: 70% stock in water, EM grade, supplied in 2-ml ampoules (Polysciences Inc., Warrington, PA) and stored at 4°C.
4. Methanol: store at room temperature.
5. Triton X-100.

### *Other Reagents*

1. Sodium borohydride: For glutaraldehyde protocol only. Sodium borohydride reduces aldehyde groups that interfere with the final signal by autofluorescing. Store light tight at room temperature as an *anhydrous* powder, and prepare working solution in CB *immediately* prior to use (within 1 min).
2. PBS/BSA/azide: PBS containing 1% BSA (Fraction V, Boehringer-Mannheim Corp., Indianapolis, IN) and 0.1% sodium azide.

## **A. Methanol Protocol**

This protocol is very quick and ideal for a “look see” preparation. Some researchers use straight methanol, whereas others do not chill it. As trace calcium ions may be present in the methanol that could affect microtubules, the authors add 5 mM EGTA, hence the methanol concentration is diluted to 95%.

1. Prepare and chill a solution of 95% methanol with 5 mM EGTA to  $-20^{\circ}\text{C}$ .
2. Transfer this solution to a chilled beaker or suitable container.
3. Drain medium from cells and wash thoroughly in prewarmed PBS.
4. Remove the coverslip from the chamber and plunge it into methanol. Incubate for 10 min without agitation.
5. Remove coverslip and submerge it immediately in PBS. Change PBS twice. Proceed with antibody application (see Section VII,E).

## **B. Formaldehyde–Triton Protocol**

1. Within 2–3 hr of use, prepare a 4% formaldehyde solution by mixing 1 part 16% formaldehyde stock with 3 parts 1.3× CB, cap tightly.

2. Warm PBS, formaldehyde solution, and  $1\times$  CB buffer to  $37^{\circ}\text{C}$ .
3. Prepare 0.1–0.5% Triton X-100 solution in prewarmed  $1\times$  CB buffer.

*Note:* As Triton is viscous, cut the end of the disposable pipette tip to assist flow. To ensure accurate measuring, wipe off excess Triton on the outside of the tip before transferring it to the CB. Prewarming the CB will facilitate detergent dissolution and its exit from the tip.

4. Transfer the formaldehyde solution to a clean fixation container.
5. Drain medium from cells and rinse twice with warm PBS.
6. Remove coverslip from chamber dish and place it cell side down on the surface of the formaldehyde solution. Incubate with gentle rocking for 10 min at room temperature. Ensure that the coverslip remains floating. If the coverslip does not float, turn it cell side up and submerge it fully.
7. Transfer warm Triton solution to a clean fixation container.
8. Remove coverslip and rinse two to three times with PBS.
9. Place the coverslip on top of the Triton solution (or submerge as before). Incubate with gentle agitation for 2 min at room temperature. (Triton concentration, temperature, or incubation time can be varied to optimize extraction.)
10. Remove coverslip and rinse thoroughly with PBS. Proceed with antibody application (see Section VII,E).

### C. Formaldehyde–Acetone Protocol

Because the dehydrating properties of acetone make cells particularly susceptible to damage, ensure that the coverslip surface is wet at all times.

1. Chill an appropriate volume of 100% acetone to  $-20^{\circ}\text{C}$  (100 ml for a  $45\times 50$ -mm coverslip).
2. Follow the protocol for formaldehyde–Triton to the end of Step 6, (skip step 3).
3. Transfer chilled acetone to a 100-ml glass beaker or appropriate container.
4. Remove the coverslip from fixative and rinse thoroughly with PBS.
5. Plunge the coverslip into the cold acetone, rock gently at first, and then incubate without agitation for 5 min at  $-20^{\circ}\text{C}$ .
6. Remove the coverslip from the acetone and plunge it immediately into a beaker of PBS.
7. Change PBS twice and proceed with antibody application (see Section VII,E).

### D. Glutaraldehyde–Triton Protocol

1. Warm PBS and  $1\times$  CB to  $37^{\circ}\text{C}$ .
2. Within 2–3 hr of use, prepare and warm the following solutions. Solution 1: 0.5% glutaraldehyde, 0.1–0.2% Triton X-100 (see Note in Section VII,B) in  $1\times$  CB. Solution 2: 1% glutaraldehyde in  $1\times$  CB.



3. Transfer solution 1 to a fixation container.
4. Drain medium from cells and gently rinse twice with PBS.
5. Remove coverslip from chamber dish and float it on the surface of solution 1 for 1 min, rocking gently at room temperature (or submerge cell side up completely; if the coverslip does not float, turn it cell side up and submerge it fully).
6. Remove coverslip and rinse twice with  $1\times$  CB.
7. In a clean fixation container, float the coverslip cells side down on the surface of solution 2. Incubate with gentle rocking for 15 min at room temperature.
8. After 13–14 min, prepare a 0.5-mg/ml solution of sodium borohydride in  $1\times$  CB. Transfer to a clean fixation container.
9. Rinse coverslip quickly as before with  $1\times$  CB.
10. Submerge the coverslip *cell side up* in the sodium borohydride solution. Incubate for 5 min at room temperature with periodic agitation. Check regularly that the coverslip has *not* floated to the top and that the solution is in contact with the entire surface of the coverslip. (The solution should be *visibly effervescing* when the coverslip is introduced.)
11. Rinse thoroughly with PBS and proceed with antibody application (see Section VII,E).

### E. Protocol for Antibody Application

Wash the specimen thoroughly between antibody incubations. Keep the sample wet during incubations to ensure that the antibody is applied uniformly. To prevent the further dilution of antibodies, dab off excess washing solutions by tilting the coverslip and touching its corner with filter paper. Antibody incubations can be performed at 4°C overnight, at room temperature for 2–4 hr, or at 37°C for 45–60 min. Although longer incubations at lower temperature may decrease nonspecific background staining, there is usually no detectable difference among these conditions. To conserve antibodies and minimize drying out, which can lead to nonspecific interactions, present antibody solutions to the sample on Parafilm in a petri dish with risers (tooth picks) to prevent the Parafilm from contacting the bottom surface of the petri dish and place this in a humidifying chamber (soaked paper toweling can provide humidity). Before application, dilute antibodies in PBS/BSA/azide and clarify by centrifugation as detailed earlier.

1. Incubate the prepared coverslips on a shaker for a minimum of 30 min at room temperature in PBS/BSA/azide. Prewarming the solution to 37°C can improve this “blocking” step.
2. Cut a piece of Parafilm the same size as the coverslip and dispense the primary antibody solution in its center. [Volume required will depend on the

size of the coverslip. For an area 35 mm in diameter (the chamber dish aperture), use between 100 and 200  $\mu\text{l}$ .]

3. Invert the coverslip and touch its surface to the antibody droplet. Capillary action will lift the Parafilm, allowing the antibody solution to coat the coverslip surface uniformly. (For small coverslips, place the antibody droplet on a large piece of Parafilm in a petri dish and invert the coverslip onto the droplet.)

5. Incubate coverslips in the inverted position at the desired temperature as stated earlier.

6. To remove the Parafilm, turn the coverslip cell side up (i.e., Parafilm up) and immerse in PBS. The Parafilm should float to the surface. (Small coverslips are separated from the Parafilm by flooding the petri dish with PBS/BSA/azide.)

7. Drain and reimmerse in fresh PBS/BSA/azide.

8. Repeat steps 1 to 6 using the secondary antibody, keeping the sample protected from light.

9. Wash in two to three changes of PBS.

10. The sample is ready for mounting or counterstaining.

## F. Counterstaining

### 1. Staining with Fluorescent Phalloidin

Phalloidin is a fungal toxin that binds specifically to filamentous actin. It is extremely poisonous and should be handled with care. Because methanol destroys its binding site on F-actin, methanol fixation should be avoided when counterstaining with fluorescently labeled phalloidin. As stock solutions are usually stored in methanol ( $-20^{\circ}\text{C}$ ) it is necessary to dry the stock prior to use.

1. Dry the appropriate volume of phalloidin using nitrogen gas or a Speed Vac.

2. Dissolve phalloidin in PBS to a final concentration of 200 nM by vigorous vortexing.

3. Using the Parafilm method just described for antibody application, apply phalloidin to the center of the Parafilm and incubate the sample inverted for 30 min at  $37^{\circ}\text{C}$  or for 45 min at room temperature.

4. Wash twice with PBS before mounting/viewing.

### 2. Staining with Hoechst 33258

1. Prepare a 10- $\mu\text{g}/\text{ml}$  solution of Hoechst 33258 in PBS from a 10-mg/ml frozen stock in dimethyl sulfoxide.

2. As described earlier, apply Hoechst 33258 solution to Parafilm and invert a coverslip onto its surface.

3. Incubate for 5–15 min at room temperature.

4. Wash with PBS and then mount/view.

### G. Protocol for Mounting Cells

1. Prepare a stock solution of 1% (w/v) *p*-phenylenediamine (PPD) in H<sub>2</sub>O (dissolves at pH 9.5), and store light tight in 1-ml aliquots at -20°C.
2. Prepare a mounting medium (MM) solution: 50% glycerol, 1× PBS, and 0.1% PPD. Adjust pH to 7.0 or pH 8.0 for fluorescein.
3. Store excess in 0.5- or 1-ml aliquots, in the dark, at -20°C.
4. Keep protected from light. Flood the chamber dish with 1 ml of MM or, if using an upright microscope, place a small drop (50–100 μl) of MM on a clean glass slide and invert a coverslip over the droplet.
5. Cells are ready to view. If the sample is to be stored for future observation (up to 6 months), seal the edges of the coverslip with nail polish.

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