18

Guiding Principles of Specimen Preservation for Confocal Fluorescence Microscopy

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INTRODUCTION

Traditionally, biologists have been confined to transmission electron microscopy (TEM) and light microscopy (LM) in order to correlate biochemical and molecular data with morphology. Electron microscopy (EM) provides fine ultrastructural detail but is limited to the study of cellular structures that react with electron dense stains deposited in fixed specimens. Immunogold labeling permits the study of non–electron-dense material, but EM sections must still be very thin to avoid problems with the penetration of the labeled antibodies and to reduce scattering of the electron beam.

The electron microscopist faces the same problem in reconstructing three-dimensional (3D) cellular structures from two-dimensional (2D) projections. In practice, the better the ultramicrotome, the more accurate the 3D reconstruction. In any event, reconstruction from serial EM sections is a laborious process.

The use of traditional LM methods, such as Nomarski optics and phase-contrast microscopy, confines one to specimens observable by transmitted light, such as *in vitro* studies involving cytoskeletal elements (Abbott, 1884; Ito, 1962; Scheetz and Spudich, 1983; Inoué, 1986; Dabora and Sheetz, 1988; Lee and Chen, 1988). The use of vital dyes and fluorescent labeling has allowed researchers to correlate functional biochemical data with structural data. The introduction of the confocal microscope has finally allowed the present generation of structural biologists to escape from Flatland. However, this instrument requires not only an imaging system, but also an object worth viewing.

Although it would probably be ideal if all biological confocal studies could be carried out on living specimens (Chapter 19, *this volume*), this is not always possible. Apart from the problem of cell movement and the nuisance of keeping cells physiologically "happy" on the microscope stage, there are additional complications in exposing such cells to antibody labeling or embedding them in antibleaching agents. As a result, the vast majority of confocal images are made from material that has been fixed, stained, and in most cases, dehydrated. However, because most LM specimen preparation techniques were developed to produce only pleasing 2D images, they are often inadequate to the task of producing specimens that retain their 3D structure.

Indeed, because of the difficulty in obtaining high contrast images from non-planar objects using conventional microscopic techniques, immunofluorescence-staining protocols that tended to flatten the specimens under study have often gained acceptance specifically because they tend to reduce the effect of out-of-focus light on the final image. This tendency has pushed the biologist to study either thin cells or cells grown under conditions that are not optimal for the expression of the full phenotype of the cell.

Confocal fluorescence microscopy extends the value of these fluorescence-labeling techniques because its ability to exclude out-of-focus information from the image data permits the acquisition of 3D intensity data sets that can be viewed as 3D images. The purpose of this chapter is to discuss methods for ensuring that the specimens from which such data are acquired maintain the 3D structure they had *in vivo*.

We have studied the Madin–Darby canine kidney (MDCK) cell line grown on Costar polycarbonate membrane filter supports. The filter supports have the disadvantage of being opaque, but they do allow the cells to be supplied with nutrients in a more physiological way. Under these growth conditions, the basal membrane has access to nutrients at all times, somewhat reproducing growth conditions *in vivo*. MDCK cells form a more completely polarized monolayer when grown on the membrane filters (Bomsel *et al.*, 1989).

These studies have asked specific questions about cellular organization both *in vitro* and *in vivo* during the formation of an epithelial monolayer. This chapter reflects the lessons learned while attempting to study these cells under growth conditions that are not very amenable to study by immunofluorescence methods.

We will begin with a description of fixatives and fixation methods. This will be followed by a section that explains how to prepare and use the two fixatives that we have found most useful (glutaraldehyde and pH shift/formaldehyde) and that also describes mounting and staining procedures. We will then describe how these procedures were evaluated and conclude with some general comments on the subject.

This chapter will discuss many of these methods and address some of the potential pitfalls of specimen preparation.

CHARACTERISTICS OF FIXATIVES

The first problem about fixatives is their name, as it may give the impression that treatment with fixatives really does "fix" or render immovable the macromolecular components of the cell. In fact, most fixation protocols are really just chemical or physical processes that cause metabolism to stop and that preserve microstructures down to some size level by destroying other structures, such as enzymes, which have sizes below that level. The best fixation for a given experiment is that which does the best job of preserving structure down to the level required.

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No fixation protocol is perfect, but its imperfection is often masked by the fact that the specimen dimensions in the plane of the image are maintained by the adherence of the specimen to the glass substrate. As a result, shrinkage in the *z*-direction is often pronounced. Such shrinkage is particularly serious when it occurs on specimens to be viewed in the confocal microscope, which allows one to obtain information in the *z*-direction.

There are three general types of fixation protocol available for biological specimen preparation. **Chemical fixatives** fix tissue either by **coagulating** proteins or by chemically **crosslinking them**. The other two methods of fixation, **freeze substitution** (Steinbrecht and Zierold, 1987) and **microwave fixation** (Jackson, 1991) will not be considered here.

The *coagulating* fixatives, such as ethanol, methanol, or acetone, fix the specimen by rapidly changing the hydration state of the cellular component. Proteins either coagulate or are extracted during this process. Coagulating fixatives are popular, as they are easy to apply reproducibly and tend to preserve the antigen recognition sites for immunolabeling very well. The major problem with the use of coagulating fixatives in confocal microscopy is the profound shrinkage of the specimen that these fixatives produce. We have found that methanol will shrink cell height by 50% during the fixation. This artifact renders the spatial information obtained by confocal microscopy inaccurate and should not be depended on, especially when the data sets are to be used for 3D reconstruction. It is also important to note that commercial preparations of formaldehyde often contain significant amounts of methanol as a stabilizing agent, and this can induce the same shrinkage artifact.

Commonly used **crosslinking** fixatives include glutaraldehyde, formaldehyde, and ethylene glycol-*bis*-succinimidyl succinate (EGS). These fixation chemicals form covalent crosslinks that are determined by the active groups in each compound.

The ideal fixative should penetrate tissues quickly, act rapidly, and preserve the cellular structure before the cell can react to produce structural artifacts. Unfortunately, no single agent has emerged as the ideal fixative and a pragmatic approach to the use of a particular fixative is determined by the experimental requirements. Although tissue fixation is incompletely understood, an understanding of the characteristics of chemical fixatives can provide a rational approach to their use.

Glutaraldehyde

Since its introduction in 1962, glutaraldehyde has been used extensively for EM specimen preparation (Sabatini *et al.*, 1962). This diadehyde is composed of five carbons with a molecular weight of 100.12. Extensive evaluation of the preservation properties of glutaraldehyde by EM has shown that it preserves subcellular structures well (Sabatini *et al.*, 1963, 1964; Barrnett *et al.*, 1964). Glutaraldehyde has been less popular as a fixative for fluorescence microscopy because it renders tissue autofluorescent and destroys antibody-binding sites. Fortunately, the autofluorescence can usually be adequately attenuated by post-treating samples with $NaBH_4$ (Tagliaferro *et al.*, 1997). Recent work utilizes ethanol extraction to remove autofluorescent chemicals or Sudan black to quench autofluorescent signals (Baschong *et al.*, 2001).

The chemistry of glutaraldehyde fixation is complex and not fully understood. Glutaraldehyde forms a Schiff's base with amino groups on proteins and polymerizes via Schiff's base catalyzed reactions (Johnson, 1985; Tashima *et al.*, 1987). The ability to polymerize allows glutaraldehyde to form extended crosslinks (Meek and Chapman, 1985). Glutaraldehyde reacts with the ε-amino group of lysine and will react with the α-amino group amino acids. It will also react with tyrosine, tryptophan, histidine, phenylalanine, and cysteine (Hayat, 1989). While glutaraldehyde fixes proteins rapidly, its main disadvantage is its relatively slow rate of penetration into the cell compared to formaldehyde. Other problems associated with glutaraldehyde fixation are the propensity of many cells to form membrane blebs as they die (as is common to all the aldehyde fixatives) and its inability to crosslink neutral lipids, DNA, or RNA (Hopwood, 1975). Not all proteins are inactivated by glutaraldehyde, and some proteins can still be extracted from fixed tissue (Blanchette-Mackie and Scow, 1981; Hayat, 1989).

Glutaraldehyde is supplied commercially as a 25% or 8% solution. Commercial preparations of glutaraldehyde may contain significant amounts of impurities, which can affect the reproducibility of the fixation method or induce fixation artifacts. In our experience, it is important to use only those commercial preparations that list the impurities. We have tended to use the glutaraldehyde solutions supplied by Polysciences (Warrington, PA) and have noted satisfactory reproducibility between commercial lots.

As the glutaraldehyde solution ages, it polymerizes and turns yellow. Because this changes the reproducibility of the fixation and can produce artifacts, it is important to identify old solutions and to store the fixative so as to inhibit polymerization (Hayat, 1989). As the polymerization rate increases with temperature and at high pH, it can be minimized by storing both unbuffered glutaraldehyde and working solutions at −20°C. These solutions should be thawed only once and used the same day.

One useful way to monitor the quality of glutaraldehyde is to measure the absorbance of a 0.5% solution at 235 nm and 280 nm. Pure glutaraldehyde has an A235/A280 ratio of less than 0.2. Artifacts tend to occur with ratios of >2.0 (Bowers and Maser, 1988).

A variety of buffers and co-fixatives can be used with glutaraldehyde. The advantages and changes in fixation chemistry that occur when it is used in combination with other fixatives have been reviewed elsewhere (Hayat, 1981; Hayat, 1986).

Formaldehyde

As mentioned above, commercial solutions of formaldehyde contain significant amounts of methanol. Formaldehyde fixation for confocal microscopy should be prepared fresh from paraformaldehyde dissolved in water (see protocol described below). Formaldehyde (MW 30.0) crosslinks proteins by forming methylene bridges between reactive groups. The rate-limiting step is a deprotonation of amino groups, hence the pH dependence of the crosslinking reaction. Other functional groups that are reactive with formaldehyde include amido, guanidine, thiol, phenol, imidazolyl, and indolyl groups (Hayat, 1989). When lysine is added to the fixation buffer, it can participate in the crosslinking reaction, and this is the basis for its inclusion in the paraformaldehyde–lysine–periodate fixation first described by McLean and Nakane (1974).

One advantage of formaldehyde as a fixative is its ability to crosslink nucleic acids. This makes it the preferred fixative for *in situ* hybridization. This aldehyde does not crosslink lipids and, in some cell types, it produces extensive vesiculation of the plasma membrane. In some cases, small amounts of $CaCl_2$ in the fixation buffer have been shown to stabilize the plasma membrane during fixation. However, labile structures such as microtubules are not well preserved by formaldehyde at physiologic pH (Sato *et al.*, 1976; Wild *et al.*, 1987). Observation of the nucleus by phase-contract microscopy during formaldehyde fixation reveals marked changes in the size and shape during fixation (J. DeMey, personal

communication). During the fixation process, the nucleus oscillates, alternating between swelling and shrinking. It is not clear what happens to the nuclear contents during this time.

The rate of protein crosslinking is slower with formaldehyde than with glutaraldehyde, however, formaldehyde penetrates cells 10 times faster (Hopwood, 1967). Attempts have been made to try to optimize fixation by mixing glutaraldehyde with formaldehyde. The idea is to take advantage of the rapid penetration of formaldehyde into the tissue and the high speed of glutaraldehyde crosslinking (Karnovsky, 1965). Although what happens to the fixation chemistry when these mixtures are employed is not clearly understood, in our experience Karnovsky's fixative has been useful for some preparations and appears to preserve the 3D structure well.

EGS is another bifunctional crosslinking agent that reacts with primary amino groups and with the ε-amino groups on lysine. A major advantage of this fixative is its reversibility. The crosslinks are cleavable at pH 8.5, an attractive feature because reversible crosslinking may be used to restore antigen binding sites (Abdella *et al.*, 1979). This fixative is useful for membrane-bound proteins, but its limited solubility in water makes its performance highly variable. Sulfated forms of this crosslinking agent [ethylene glycobis (sulfo-succimidylsuccinate)] (Pierce Co., Pierce, IL) are available and are water soluble (Abdella *et al.*, 1979). We have not tried this particular crosslinking agent as yet but it is also cleavable.

FIXATION STAINING AND MOUNTING METHODS

The fixation methods described below have been optimized for the confocal fluorescence microscope in studies in which MDCK cells have been grown on opaque filter supports (Bacallao and Stelzer, 1989). The cells grow to a uniform height of 18 µm and form a dense monolayer under the growth conditions we used. These fixation and staining protocols have been tested using the criteria described in the next section.

Glutaraldehyde Fixation

Stock Solutions

- 8% glutaraldehyde EM grade (Polysciences, Warrington, PA).
- 80 m*M* Kpipes, pH 6.8, 5 m*M* EGTA, 2 m*M* $MgCl_2$, both with and without 0.1% Triton X-100 depending on the target antigen; use Triton if you intend to stain cytoskeletal proteins.
- Phosphate-buffered saline without $Ca^{2+}MG^{+}$ (PBS–).
- Phosphate-buffered saline without $Ca^{2+}MG^{+}$, pH 8.0.

Preparation of the Stock Solutions

EM grade glutaraldehyde was obtained from Polysciences. It is supplied as an 8% aqueous solution. When a new vial is opened, the glutaraldehyde is diluted to 0.3% in a solution of 80 m*M* Kpipes, pH 6.8, 5 m*M* EGTA, 2 m*M* $MgCl_2$, 0.1% Triton X-100. The aliquots are stored at –20°C. Prior to each experiment, a fresh aliquot is used soon after thawing. These aliquots are never frozen again or reused because this causes a loss of efficacy. Bring up the pH of the PBS by adding a few drops of 6 *N* NaOH to PBS.

Fixation Protocol

1. Warm 100 mL of 80 m*M* Kpipes, pH 6.8, 5 m*M* EGTA, 2 m*M* $MgCl_2$ without Triton X-100 to 37°C in a beaker.
2. Pour off the media in the apical well of the Costar filter. Dip the entire filter plus filter holder into this modified 80 m*M* Kpipes buffer for 5 s.
3. Transfer the filter to the 6-well plate supplied with the polycarbonate filters to permit convenient fixation and washing steps.
4. Fix the cells for 10 min with 0.3% glutaraldehyde +0.1% Triton X-100 at room temperature. The glutaraldehyde fixative is added to both the apical (2 mL) and basal (3 mL) portions of the filter. During all the incubation steps and washes, the 6-well plate is agitated on a rotary shaker.
5. During the fixation period, weigh out 3- to 10-mg aliquots of fresh $NaBH_4$. The $NaBH_4$ powder is then stored in 50 mL sterilized conical tubes with screw caps. The $NaBH_4$ should be kept in an anhydrous state, preferably under dry nitrogen gas because it is a very strong reducing agent and, when combined with water, hydrogen gas is released. **Explosions in the laboratory setting have been reported so this agent should be used with care.**
6. Aspirate the fixative, and dip the entire filter successively in three separate 100 mL beakers containing PBS–.
7. Add PBS–, pH 8.0 to the $NaBH_4$ to make a final concentration of 1 mg/mL. Add 3 mL of this solution to the apical portion of the cell and 4 mL to the basal chamber. Incubate 15 min at room temperature. You should see gas bubbles in the solution during this step. Repeat this step two more times using freshly dissolved $NaBH_4$. The adjustment of the pH to 8.0 increases the half-life of $NaBH_4$ in solution. This step is essential to decrease the autofluorescence of the glutaraldehyde-fixed cells.
8. Wash the cells with PBS– by dipping the preparation in three beakers containing PBS–. Return the filters to the 6-well plate with PBS– bathing the apical and basal side. The filter is now ready for immunofluorescence staining.

pH Shift/Formaldehyde Fixation

The pH shift/formaldehyde method was first used for fixing rat brain, in which it showed excellent preservation of neuronal cells and intracellular compartments (Berod *et al.*, 1981). This technique applies the formaldehyde to the tissue twice: once at near physiological pH to halt metabolism, and then again at high pH, where the crosslinking action of the fixative is more effective.

Stock Solutions

- 40% formaldehyde (Merck) in H_2O.
- 80 m*M* Kpipes, pH 6.8, 5 m*M* EGTA, 2 m*M* $MgCl_2$.
- 100 m*M* NaB_4O_7 pH 11.0.
- Phosphate-buffered saline without Ca^{2+}/Mg^{2+} (PBS–) pH 8.0.
- Phosphate-buffered saline without Ca^{2+}/Mg^{2+} (PBS–) and both with and without 0.1 % Triton X-100.
- Premeasured 10-mg aliquots of dry $NaBH_4$.

Preparation of the Stock Solutions

Preparation of the formaldehyde stock solution is based on the description by Robertson and colleagues (1963). Forty grams of paraformaldehyde $(CH_2O)_n$; MW 30.3 is added to 100 mL of H_2O. While continuously stirring, the mixture is heated above to 70°C. A few drops of 6 *N* NaOH are added to dissolve the formaldehyde, but do not allow the solution to boil. The stock solution is divided into aliquots and stored at –20°C. Prior to use, aliquots are thawed by warming in a water bath. The formaldehyde is diluted to 2% to 4% in both the Kpipes and sodium borate buffers. For our purposes, a 3% solution of formaldehyde was adequate for preserv-

ing both the structure and antigenic determinants on a wide variety of cell organelles. The pH of the Kpipes buffer is brought to 6.5 with 1 *N* HCl after the formaldehyde has been added.

PBS–, pH 8.0 is made by adding a few drops of 6 *N* NaOH to normal PBS, made without calcium or magnesium; 100 m*M* NaB_4O_7 is titrated to pH 11.0 by adding 6 *N* NaOH to the buffer.

Fixation Protocol

1. Pour off the media in the apical well of the filter.
2. Dip the filters in 80 m*M* Kpipes solution pre-warmed to 37°C.
3. Add 3 mL of 3.0% formaldehyde in the 80 m*M* Kpipes solution to the basal chamber of the 6-well dish, and add 2 mL of this solution to the apical surface of the cells. Incubate the cells with agitation on a rotary table for 5 min at room temperature.
4. Aspirate the formaldehyde/Kpipes solution; then add 3 mL of 3% formaldehyde in 100 m*M* NaB_4O_7, pH 11.0, to the basal side and 2 mL to the apical side of each filter. Incubate with agitation on a rotary table for 10 min at room temperature.
5. Weigh out two 10-mg aliquots of $NaBH_4$ for each filter and store in a conical tube with a screw cap.
6. Aspirate the fixation solution. Wash the filters by successively dipping the filters in three beakers containing 100 mL of PBS–.
7. Dissolve each aliquot of $NaBH_4$ in 10 mL PBS–, pH 8.0 (final concentration of $NaBH_4$ should be 1 mg/mL). Vortex the solution briefly, and add it to the apical (2 mL) and basal (3 mL) portions of the filters. Incubate 15 min while shaking the filters on a rotary table. Repeat this step one more time using a fresh solution of $NaBH_4$ in PBS–, pH 8.0.
8. Wash the filters by successively dipping the filters in three beakers containing 100 mL of PBS–. The filters can be stored overnight at 4°C with PBS–/0.1% NaN_3.
9. The sample is permeabilized by washing with PBS (–) + 0.1% Triton X-100.

Immunofluorescence Staining

1. Cut the filter from its plastic holder. Be sure to note which side of the filter has the cells layered on it! Cut the filter into four squares using a sharp scalpel while keeping it wet with PBS. To ensure that the cell side of the filter can be readily identified, we routinely cut a slit in the upper right corner of the filter. The filter is cut into squares because this tends to give a flat field of cells after the filter has been mounted. Dividing a filter into quadrants with one rounded edge causes the filter to ripple during mounting.
2. Wash the filter squares in PBS– containing 0.2% fish skin gelatin (FSG), which is used as a carrier protein instead of albumin in this protocol (Sigma, St. Louis, MO) and the appropriate percentage of detergent. The amount of detergent must be determined empirically. Usually 0.1% to 0.3% Triton X-100 will suffice. Wash the filter squares in a 6-well plate. All washes are done in 4 mL of solution, at room temperature, with agitation. Unless otherwise stated, the filters are washed for 15 min after every change of washing buffer.
3. Place a 50 µL drop of the first antibody diluted in PBS– containing 0.2% FSG on a piece of parafilm on the bottom of a petri dish.
4. Place a filter square, cell-side down, on the antibody solution. Place a piece of wet Whatman filter paper in the petri dish, well away from the antibody solution and then cover to form a small, humidified chamber.
5. Incubate at 37°C for 1 h (for specimens that are <10 µm thick, 35 min is an adequate incubation time).
6. Wash the filter twice with PBS– containing 0.2% FSG. Follow this with three successive washes with PBS–.
7. Wash the filter once more with PBS– containing 0.2% FSG.
8. Add the second antibody as described for the first antibody.
9. Incubate at 37°C for 1 h.
10. Wash the filters in PBS– containing 0.2% FSG twice.
11. Wash three times in PBS–.
12. Incubate once in PBS containing 0.2% Triton X-100 for 5 min.
13. Wash twice in PBS– for 5 min each time.

The filter squares are ready for mounting.

Mounting the Specimen

Place four drops of clear acrylic nail polish on a microscope slide to make corner supports for a coverslip. Each drop should be at a point corresponding to the corner of a coverslip. We use 22 × 22 mm 1.5 coverslips. Place the filter square in the center of the area demarcated by the nail polish. Take care to ensure that the cells are facing up. Put a drop of 50% glycerol / 50%PBS / 0.1%NaN_3 / 100 mg/mL DABCO (1,4-diazabicyclo [2.2.2.] octane; Sigma) on the filter. Carefully place the coverslip over the filter. Avoid trapping air bubbles in the specimen mount and make sure that the corners lie on the drops of polish. Aspirate the excess glycerol medium. Put four drops of nail polish on the four corners of the coverslip to stabilize the mount. Alternatively, shards of broken coverslips can be used as coverslip supports. Once the nail polish on the corners has dried, the entire mount can be sealed with nail polish. The specimen should be viewed within 24 h because these are not permanent mounts.

Semi-permanent samples can be made by post-fixing the filter in 4% formaldehyde dissolved in 100 m*M* Na cacodylate, pH 7.5, for 30 min at room temperature followed by quenching with 50 m*M* NH_4Cl in PBS for 15 min. Post-fixed samples can maintain excellent labeling characteristics for over 6 months when stored at 4°C or at –20°C. Figure 18.4 shows the unfortunate result of an improper mounting method. In this case, the coverslip was not adequately supported because the acrylic nail polish posts were set too far apart: clearly, attention to the details can determine the success or failure of specimen preparation.

CRITICAL EVALUATION OF LIGHT MICROSCOPY FIXATION AND MOUNTING METHODS

Immunofluorescence labeling methods have been widely employed in cell biology (Osborn *et al.*, 1980; Osborn and Weber, 1982). The major concerns when using these methods to study cell morphology have been to preserve the antigenic and structural integrity of the specimen. In general, the goal is to immobilize the antigen quickly, while preserving the cell's organization. All too frequently, the best preservation methods destroy the epitopes required for antibody binding. Because this is a particular problem with glutaraldehyde (Nakane, 1975; Cande *et al.*, 1977; Weber *et al.*, 1978), formaldehyde is often used as a fixative despite its poorer preservation.

Our initial attempts to use the "classical" fixation and mounting methods for normal epi-fluorescence microscopy yielded images that were markedly deformed in the third dimension. It became apparent that these methods had been specifically designed to shrink the cells flat to the coverslip in order to reduce the out-of-focus light in standard epi-fluorescence images. To avoid this, it became necessary to meld some of the fixation methods devel-

oped for EM with the techniques of classic immunofluorescence. This meant that, in addition to the requirements for structural and antigenic preservation, the 3D spatial preservation had to be considered as well. This matter has also been a concern for workers using scanning EM and those doing stereographic analysis of TEM images. Because samples being prepared for immunofluorescence microscopy do not have to be dried, the major concern we had was shrinkage induced by fixation or dehydration. This problem has been examined by several workers (Tooze, 1964; Boyde and Maconnachie, 1979, 1981; Lee *et al.*, 1979, 1982; Wangensteen *et al.*, 1981; Lee, 1984). Glutaraldehyde fixation has been found to induce cell shrinkage when hypertonic buffers are used. Additionally, Lee has shown that the total osmolarity of the fixative and the type of buffer used determine the extent of gross specimen shrinkage (Lee *et al.*, 1982; Lee, 1984). These findings have important implications for the preparation of tissue samples for two-photon microscopy where thicker tissue sections can be imaged.

Other fixation procedures can also affect the degree of cell shrinkage. For example, osmium tetroxide fixation causes a variable degree of volume shrinkage in erythrocytes. It has been suggested that the amount of shrinkage induced by this fixative depends on the interplay between the electrostatic interactions between charged protein particles and osmotic forces (Tooze, 1964). A recent review and study on the effect of formaldehyde fixation on cell volume found that rat liver strips shrank in length by only 3% when fixed at room temperature. The liver strips were immersed in fixative and observed by video microscopy, but the assessment of shrinkage was only measured in the length dimension. Raising the temperature to 39°C diminished the amount of shrinkage observed (Fox *et al.*, 1985). It was also noted that, in general, increasing the concentration of formaldehyde paradoxically seemed to cause swelling of the sample. This effect has not been satisfactorily explained but has been noted by other investigators (Bradbury and Meek, 1960). The shrinkage is due to the extremely high total osmolality of fixative solutions containing formaldehyde. It should be noted that a 3% solution of paraformaldehyde has a far higher osmolality (approximately 1000 mOsm) than that of a 3% solution of glutaraldehyde (300 mOsm) because of the large difference in their molecular weights (Fox *et al.*, 1985). The buffer should be optimized to be isotonic with the specimen when paraformaldehyde is used.

Our overall approach to resolve these conflicting requirements reflected a bias toward immunofluorescence studies. We started with fixation methods that still preserved the epitope binding sites for our antibody label and then tried modifications designed to be freely transferred from one system to another. An example of this occurred when the paraformaldehyde/pH shift fixation protocol was adopted to study microtubule/kinetochore interactions during mitosis in newt lung epithelial cells. While the microtubules were well preserved, it was found by differential interference contrast (DIC)–video microscopy that the chromosomes continued to move during the low-pH incubation step (A. Merdes, unpublished observations). This indicated that the cells were not immediately immobilized by the first step of the fixation and, as a result, this method was not useful for these cells in this particular study. This example points out the advantage of observing the specimen under phase contrast or DIC during fixation to detect potential artifacts.

Use of the Cell Height to Evaluate the Fixation Method

In order to generate 3D images that accurately reflect *in vivo* cellular architecture, it was necessary to employ fixation methods that minimize cell shrinkage or distortion. To study this issue, we took advantage of the ability of the fluorescent lipid analog C6-NBD-ceramide to label the plasma membrane *in vivo* (Lipsky and Pagano, 1985). The plasma membrane of MDCK cells, grown to confluence on filter supports, was labeled as described by van Meer and colleagues (1987). The height of the living cells was determined from randomly selected vertical sections using confocal *xz*-images (Fig. 18.1). The samples were then fixed and stained with probes that recognized either actin filaments or microtubules, using procedures described previously. Actin staining and microtubule staining were used as markers for the cell height in the fixed cells because these networks lie close to the plasma membrane. Furthermore, the preservation of the cytoskeletal network is particularly sensitive to fixation methodology. The mean height of the fixed cells was then determined again at randomly selected sites and the results from the two groups (fixed vs. *in vivo* cells) were analyzed by Student's *t* test. Figure 18.1(A) shows the plasma membrane labeling observed in cells labeled *in vivo* with C6-NDB-ceramide and Figure 18.1(B) shows immunofluorescence labeling of another part of the same sample after glutaraldehyde fixation. Both samples were mounted in PBS. The height and shape of both samples are quite similar. We observed that the shape of the apical dome was very sensitive to fixation artifacts and that glutaraldehyde fixation, with or without Triton X-100 in the buffer, preserved

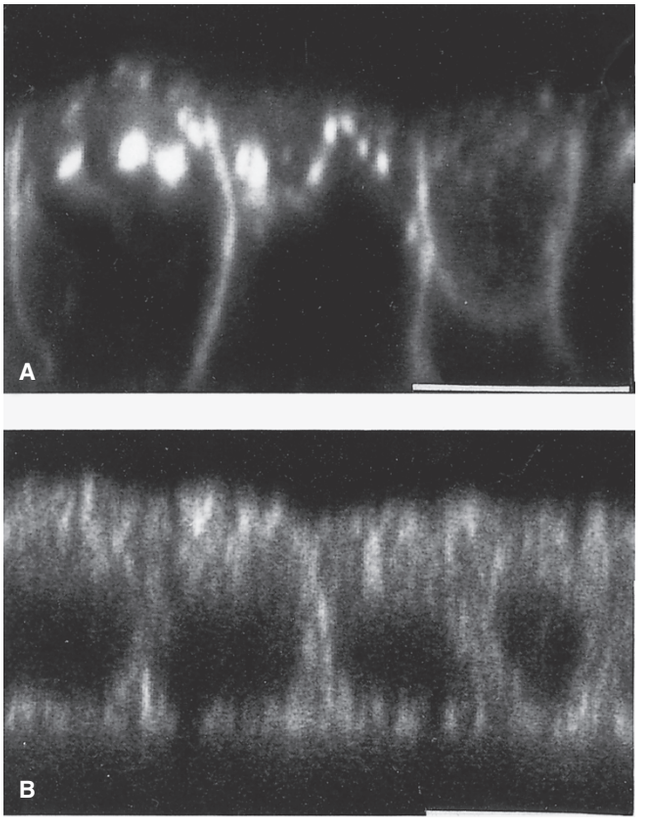

FIGURE 18.1. Vertical optical sections of MDCK cells. MDCK cells were plated on polycarbonate filters and grown for 5 days in culture. The cells have formed a columnar epithelium of fairly uniform height. (A) C6-NBD-ceramide labeling of the plasma membrane. Note the curved appearance of the apical plasma membrane. (B) Microtubule staining in glutaraldehyde-fixed cells. Note that the cell height and apical membrane curvature are roughly comparable. Bar = 10 μm.

the cell height most accurately with very little shrinkage detected as long as the buffer was isotonic.

Less than 5% increase in the cell height was caused by pH shift/formaldehyde fixation. We consider this degree of distortion acceptable in some well-defined situations. However, fixation with paraformaldehyde alone had a paradoxical effect on cell size noted above when the pH shift method was not used. Higher concentrations of paraformaldehyde (4%) dissolved in low-ionic-strength buffers were less likely to cause cell swelling. Lower concentrations of paraformaldehyde in hyperosmotic buffers either showed no change in cell size or caused shrinkage. It became apparent that no single buffer yielded optimal fixation when paraformaldehyde was used as a fixative.

Figure 18.2 shows stereo images of isolated cells fixed with methanol and then the formaldehyde/pH shift method. The methanol has caused a flattening of the area overlying the nucleus and a 10% to 20% decrease in cell height. Whenever methanol was used on confluent cells as either a fixative or as a permeabilization agent, it caused a 20% to 50% decrease in cell height. We consider it inappropriate to use this agent on samples prepared for 3D examination in a confocal fluorescence microscope.

Use of Cell Height to Evaluate Mounting Media

Shrinkage is often associated with removing biological structures from the aqueous environment and placing them in less polar solvents having a lower dielectric constant (Boyde and Maconnachie, 1979, 1981). Confocal *xz*-images were also employed to evaluate the effects of different mounting methods and mounting media. However, as all such media have different indices of refraction (η) and as the apparent specimen height measured in this way is only accurate if the specimen is immersed in a medium having the η for which the objective is corrected (see Chapter 20, *this volume*; Hell *et al.*, 1993), these measurements should be considered more comparative than quantitative.

Gross distortion is produced if the coverslip touches the specimen (Fig. 18.4). To avoid this, we suspended the coverslip using four posts of nail polish as mentioned above.

The best mounting media was found to be 50% glycerol in PBS, which showed no detectable shrinkage in fixed specimens compared to *in vivo* labeled cells. Mowoil and Gelvatol caused a 10% decrease in cell height in glutaraldehyde-fixed cells. This amount of shrinkage was considered to be significant because the apical domes, seen *in vivo*, in polarized MDCK cells were completely flattened by these mounting agents. The mounting media appeared to have an even greater effect on the shape of formaldehyde-fixed cells, probably due to the lower degree of crosslinking in formaldehyde-fixed specimens.

Well-Defined Structures Can Be Used to Evaluate Fixation Methods

A second method used to evaluate fixation was to examine structures that have a well-defined morphology. Because we had an interest in microtubule organization in epithelial cells, we examined the preservation of mitotic spindles in our sample preparations. Changes in the natural symmetry of the mitotic spindle, due

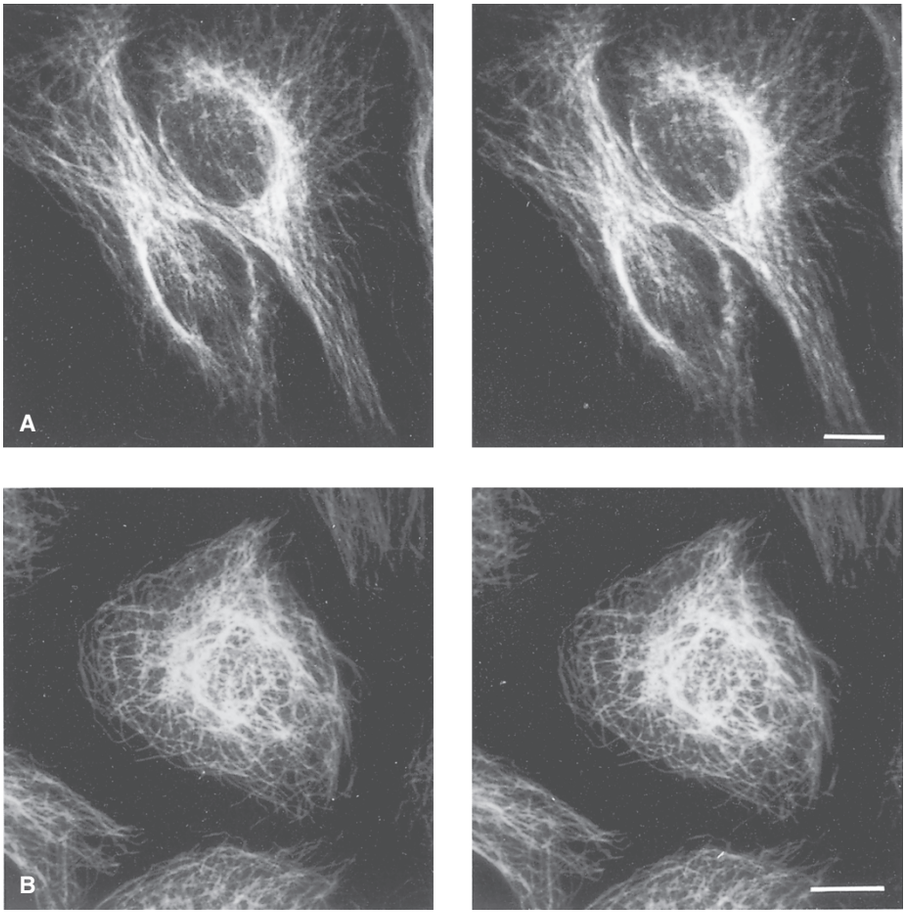

FIGURE 18.2. Stereo image of isolated MDCK cells. MDCK cells were plated at a low density, one day prior to fixation and staining. This image was reconstructed from a series of optical sections taken in the *x-y* plane from consecutive "*z*"-positions, 0.4 µm apart. No other image processing was performed. Each field is made up of 512 × 512 pixels. The image is the average of four scans per line. (A) Microtubule staining in methanol-fixed cells. Note the flattening over the nuclear region. The distance from the top of the cell to bottom is 4.1 µm. (B) Microtubule staining in cells fixed with the formaldehyde/pH-shift method. No flattening is noted and the microtubules are well preserved. The distance from the top of the cell to the bottom is 4.5 µm. Bar = 10 µm.

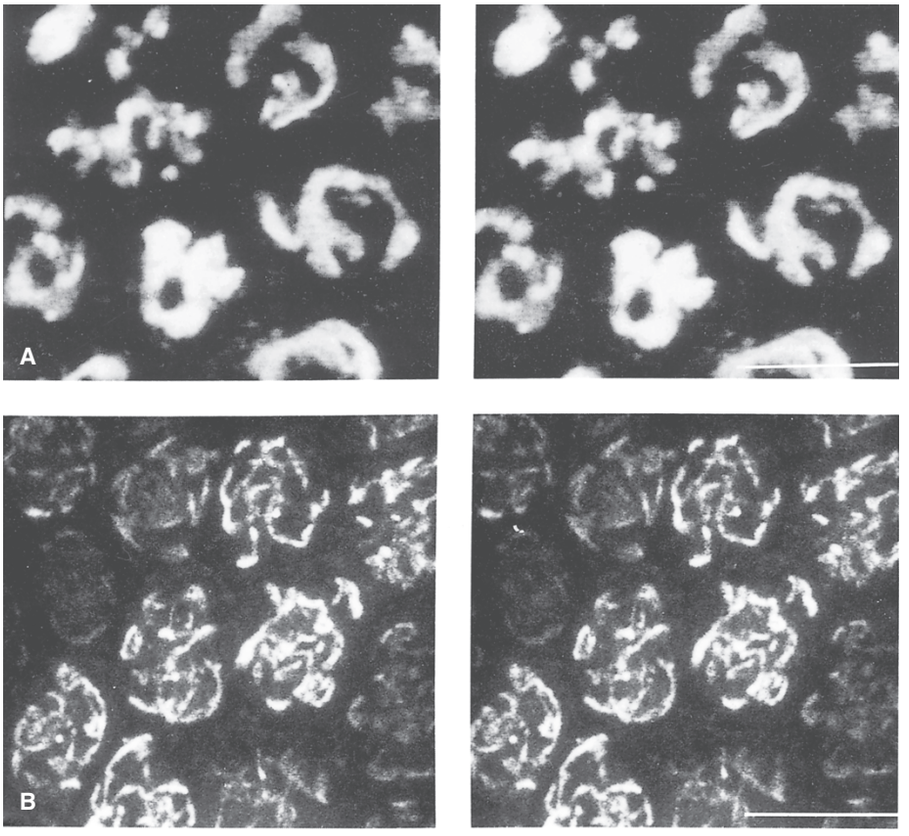

FIGURE 18.3. (A) Stereo images of the Golgi apparatus in confluent MDCK cells labeled *in vivo* with C6-NBD-ceramide made under the same conditions as Figure 18.2. Note the convoluted morphology. The distance from the top of the Golgi to the bottom is 6.4 μm. (B) Stereo images of the Golgi apparatus in confluent MDCK cells labeled by a monoclonal antibody. Cells were fixed by the formaldehyde/pH-shift method described in this chapter. Note that the overall morphology is similar to the ceramide labeled morphology. The image was generated as described above. Bar = 10 μm.

to fixation or mounting artifacts, were easily detected. Breaks in the microtubules or shortening of those within the spindle could be seen readily, and such changes were used to disqualify some fixation methods.

In general, formaldehyde fixation yielded poorly preserved microtubules, however, the formaldehyde/pH shift fixation method gave acceptable microtubule preservation of the mitotic spindles. When compared directly with glutaraldehyde fixed cells, the formaldehyde/pH shift method was not so good, but it was an acceptable compromise. In direct comparisons, the staining of formaldehyde/pH shift–fixed specimens was slightly lower in intensity than that in specimens fixed with formaldehyde alone. However, we found it was useful for double-immunofluorescence labeling with antigenic epitopes that were destroyed by glutaraldehyde fixation. The periodate–lysine–formaldehyde fixation described by McLean and Nakane (1974) did not preserve mitotic spindles well.

Comparison of *In Vivo* Labeled Cell Organelles with Immunolabeled Cell Organelles

Sometimes membrane structures can be distorted even though the fixation method does not affect cell height. As noted above, light microscopic observation of cell nuclei during fixation with formaldehyde revealed marked changes in nuclear size and shape during the pH 6.5 step of the fixation that showed it was unsuitable for the examination of nuclear membrane antigens, at least at this tonicity.

The use of vital fluorescent dyes such as rhodamine 123,3,3′-dihexyloxacarbo-cyanine (DiOC6) and C6-NBD-ceramide permits comparison of the effects of subsequent fixation on the morphology of the mitochondria, rough endoplasmic reticulum (RER), and Golgi apparatus, respectively (Walsh *et al.*, 1979; Terasaki *et al.*, 1984; Lipsky and Pagano, 1985; see Chapter 19, *this volume*).

MDCK cells undergo striking changes in the morphology of their Golgi apparatus during the formation of a polarized epithelium (Bacallao *et al.*, 1989). The morphology in the final polarized state was examined *in vivo* using the fluorescent lipid analog, C6-NBD-ceramide. The morphology seen *in vivo* was very similar to that in samples fixed and stained using a Golgi-specific monoclonal antibody generously supplied by Dr. M. Bornens (Fig. 18.3) and to that seen in non-ciliated epithelial cells, as determined by an analysis of thick sections viewed at low magnification in the high-voltage electron microscope (HVEM) (Rambourg *et al.*, 1989). Both glutaraldehyde fixation, and the pH shift/formaldehyde fixation protocol, preserved the morphology of the Golgi apparatus well.

GENERAL NOTES

The repeated use of borohydride in these fixation protocols was found to decrease endogenous cellular fluorescence significantly. Other quenching agents, such as ammonium chloride and lysine, were also tried, but borohydride worked best. This was a crucial obstacle to overcome in our work because our specimens had high cell densities when the MDCK cells grew to confluence on mem-

brane filter supports. Initially, high endogenous background fluorescence caused our images to have poor contrast, obscuring important details.

These fixation methods also work well with thinner cells grown on coverslips, but some shortening of the fixation time is necessary. Typically, a 4-min fixation in the pH 6.5 buffer and an 8-min fixation in the pH 11.0 buffer worked well with glass-grown cells. Although in some tissues there is a tendency for formaldehyde to induce vesiculation of cell membranes, this artifact was not observed in all tissues.

Both saponin and Triton X-100 have been used as permeabilization agents in the formaldehyde fixation method. Triton X-100 has been most effective when used after the fixation was complete, while saponin worked best when included with the borate buffer. NP-40 worked very well for specimens in which the microtubules were stained with monoclonal antibodies to α and β tubulin.

We have used DABCO at a concentration of 100 mg/mL as an antibleaching agent (Langanger *et al.*, 1983). Yellowing of the specimen occurs 1 month after mounting when DABCO is included with the mounting media, but this does not seem to produce a significant change in the images produced from such samples. A recent paper suggested that 5 *M* NaN_3 is a better antibleaching agent than DABCO (Bock *et al.*, 1985), however, in our experience, we found the reverse (Merdes *et al.*, 1991). *n*-Propylgallate has been tried as an antibleaching agent but in our hands it caused a dimming of the fluorescent signal. We did not use *p*-phenylenediamine as an antibleaching agent because this agent destroys the sample over time (Langanger *et al.*, 1983). Some investigators have tried to make mixtures of antibleaching agents such as 5% *n*-propylgallate-0.0025% *p*-phenylenediamine dissolved in glycerol. Another mixture suggested by Peter Hahn (Thomas Jefferson University, Philadelphia, PA) is a 0.25% *p*-phenylenediamine, 0.0025% DABCO, 5% *n*-propylgallate dissolved in glycerol with a pH value of approximately 7.6. These investigators did note some increase in background signal, however (see also Chapter 39, *this volume*).

The pH of the mounting media can be an important parameter to consider, especially when fluorescein is used as a fluorophore. The fluorescent emission of fluorescein conjugates increases up to pH 10. For additional insights on the effect of pH on fluorescent compounds, the readers are referred elsewhere (Hiramoto *et al.*, 1964; Klugerman, 1965).

Labeling Samples with Two or More Probes

One of the unique aspects of 3D imaging is that it gives one the power to determine spatial relationships. However, this can also pose problems if one can see only the stained structure. For example, in our early work using C6-NBD-ceramide to study the Golgi morphology, we found ourselves dissatisfied with the images. One frequently wanted to know where the Golgi apparatus was located with reference to other cellular structures (see Fig. 18.3). Sometimes a reference image can be provided by detecting and displaying the light scattered back by optical inhomogeneities in the specimen. This signal can be obtained without interfering with the collection of the fluorescence signal (Pawley *et al.*, 1993; Chapter 2, *this volume*) but, as doing so requires equipment that is only now becoming generally available, it has been more common to image other cellular structures using a second fluorescent dye.

Our early attempts to label these structures simultaneously led to unforeseen difficulties. The first major pitfall was the finding that fluoroscein isothiocyanate (FITC) and rhodamine could not be used simultaneously in combination with the filter sets then available to us. Although the cutoff filters were supposed to eliminate signals above 530 nm, we were unable to separate cleanly the FITC image from the rhodamine image. This was due in part to the fact that the confocal microscope we used had an argon-ion laser with lines at 528.7 and 476.5 nm, so even when the 528.7 line is used, FITC is still slightly excited and the cutoff filters cannot completely eliminate the FITC signal. At the shorter wavelength, we experienced problems with fluorescence energy transfer because the light emitted from the FITC was exciting the rhodamine. This problem was partially overcome by combining FITC with Texas Red for double-labeling experiments. Each of the two laser wavelengths was used to excite a single fluorophore, but using 528.7 nm excitation, a higher cutoff filter (580 nm) could then be used to block out the FITC signal. There were no detectable problems with fluorescence energy transfer with this combination; however, it did present another difficulty. Although the laser we used has lines available at 528.7 and 476.5 nm, the power at these wavelengths varies markedly with time and temperature, and, in addition, if a double dichroic is not used (Chapter 9, *this volume*), changing the filters to select a different wavelength can produce mis-registration between the two images. Both of these situations can produce sets of images that are not matched in intensity, and the detector gain must be adjusted to "normalize" them.

Another useful combination was Lucifer Yellow and Texas Red, which were used to study the endocytic compartments in filter-grown MDCK cells *in vivo*.

Using a krypton–argon laser, which has lines at 488 nm and 567 nm, has reduced difficulties with bleed-through. The 567 nm line is closer to the excitation maximum of Texas Red and, with the proper configuration of cutoff filters, allows the user to label specimens with FITC and Texas Red (see Chapters 16 and 36, *this volume*).

We have successfully double-labeled specimens with FITC and rhodamine/Texas Red using a helium–argon (He-Ar) laser. One of the cellular components is labeled with two secondary antibodies having the same antigenic specificity. The secondary antibody mixture contains a 1 : 4 molar ratio of rhodamine/Texas Red conjugated antibodies. We found that there was no bleed-through when the FITC signal was imaged. However, using the 514 nm line gave an excellent signal from the rhodamine/Texas Red mixture (Bacallao and Garfinkel, 1994).

Some consideration must be given prior to the experiment as to what structures will be labeled with which fluorophore. In our experience, perhaps because of the higher quantum efficiency of the detector or the lower diffraction limit, FITC appears to give images with better contrast than does Texas Red, so structures with fine detail (e.g., microtubules) tend to yield a better image when stained with FITC (Fig. 18.4). In samples that have been labeled with both FITC and Texas Red, the FITC image must be obtained first, followed by the Texas Red image, because FITC bleaches rapidly even with antibleaching agents present. Most microscopes now permit the simultaneous acquisition of images produced by these fluorophores (Chapters 2 and 9, *this volume*).

Triple Labeling

One promising method for triple labeling involves the use of immunogold, silver-enhancement labeling techniques (Lackie *et al.*, 1985; Scopsi and Larsson, 1985; Bastholm *et al.*, 1986; Birrell and Hedbert, 1987; Danscher *et al.*, 1987). This labeling method has been used successfully to stain cell adhesion plaques (Paddock, 1989, 2002).

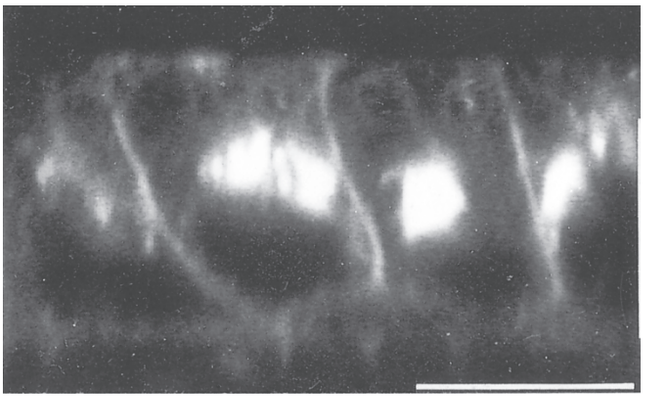

FIGURE 18.4. Deformation of a sample due to improper mounting. A confluent monolayer of MDCK cells was labeled *in vivo* with C6-NBD-ceramide. The coverslip was placed on the acrylic spacing mounts incorrectly. The apical surface has been completely flattened. The basolateral membranes are no longer vertical probably due to shearing. Bar = 10 µm.

A third label can be added to a double-labeled fluorescent sample using gold-conjugated antibodies, which can be imaged in the backscattered light mode (Pawley *et al.*, 1993; Linares-Cruz *et al.*, 1994). Image-processing techniques should allow one to overlay all three images. Once again, the cellular structures to be studied should be matched with the particular labeling method employed. In our initial attempts to study mitotic spindles using immunogold labels, the gold was so dense that it acted as a mirror, preventing us from obtaining an image below the upper half of the spindle.

We have routinely used three or more fluorescent probes on single samples to image various intracellular compartments using the Zeiss 510 confocal microscope equipped with an Enterprise laser (Spectra Physics, Mountain View, CA), two helium–neon (He-Ne) lasers and a Kr-Ar laser. Laser lines at 353 nm, 383 nm, 488 nm, 512 nm, 563 nm, and 650 nm are available for imaging. Appropriate emission filter sets are available on this system that make four-color fluorescence imaging routine. We have found it to be particularly useful to routinely label nuclear compartments with DAPI or Hoescht 3314. Computer programs designed to measure the extent of co-localization between two or more labeled marker proteins give correlations greater than 0.5 for any two markers, even if there is no visual evidence for co-localization. This is because the nucleus comprises approximately 50% of total cell volume, so any cytoplasmic protein excluded from the nucleus is automatically constrained to the other 50% of the total volume. By routinely labeling the nuclear compartment, we can make masks that remove the nucleus from the analyzed volumes to obtain realistic correlation coefficients. A computer program that give percentage of co-localization and correlation coefficients is under development and is called CORR3D (Christopher Constantine, manuscript in preparation). It will be available as shareware at http://www.nephrology.iupui.edu.

Preparation of Tissue Specimens

Preparation of tissues for examination by confocal microscopy is complicated by problems of fixative penetration, the heterogeneous cellular composition of tissues, and the presence of the extracellular matrix. Tissue slices are fixed by two general approaches: immersion and perfusion. With immersion fixation, the tissue is dissected, cut into small pieces, and immersed in the fixation solution. The dissection must be performed carefully in order to avoid damaging the specimen. Penetration of the fixative is dependent on the thickness of the specimen and the type of fixative (formaldehyde penetrates faster). During immersion fixation, cells are exposed to anaerobic conditions; a gradient of fixative concentration means that deeper regions are often fixed less well than superficial regions.

Fixation of tissues by perfusion is preferable in most examples because the fixative reaches all of the tissue more rapidly (Ericsson and Biberfeld, 1967; Fahimi, 1967; Petersen, 1977; Nowell and Pawley, 1980), and the cells are less likely to develop anoxic damage (Rostgaard *et al.*, 1993). The perfusion pressure must be carefully controlled because otherwise this can be a source of tissue damage. A recommended list of organ-specific perfusion pressures has been reported by Hayat (1989). We have used either diluted Karnovsky's fixative or paraformaldehyde fixative with a perfusion pump to fix kidneys with satisfactory morphological preservation. The osmolality of the paraformaldehyde fixation buffer must be optimized depending on the region of the renal tubule one wishes to study (Hayat, 1989).

Preparing tissue for confocal microscopy brings separate concerns. Because excitation wavelengths are shifted toward infrared, it is possible to image deeper into tissue (Brakenhoff *et al.*, 1996; Soeller and Cannell, 1996). We routinely image 100 µm thick vibratome sections labeled with a variety of antibodies and fluorescent-tagged lectins. The preparation method for labeling thick sections is provided here.

Labeling Thick Sections

1. **Perfuse-fix** anesthesized mice with 4% paraformaldehyde (PFA) (made in 1× PBS). Flush ice cold 1× PBS through vasculature via left ventricle before PFA perfusion.
2. **Cut** vibratome sections of kidneys (50–200 µm thickness).
3. **Wash** sections at room temperature, 2 to 4 h, in 1× PBS (vigorously on orbital shaker/rocker). We use 14 mL in a 15 mL Falcon tube (air bubble helps with agitation). Carefully transfer wet sections with soft bristle brush or one tip of forceps — don't squeeze tissue!
4. **Block**: incubate tissue sections in ~200 µL blocking buffer — sections should be in small PCR tubes (blocking buffer is 0.1% to 1% Triton X-100*, 1% to 2% BSA, 1× PBS, make fresh). Put back on rotator/shaker for 1 to 2 h.
5. Add **primary antibody** diluted in blocking buffer (we use 200 µL total volume in small PCR tubes) — place labeled tubes in dry 50 mL Falcon tube and rotate overnight on rotator/rocker 4°C (or 4+h at room temperature, depending on thickness of sections).
6. Next morning repeat **wash** (see step 3).
7. Incubate in fluorescent-labeled** **secondary antibody** diluted in blocking buffer (same conditions as step 5 above). Also good time to add fluorescent-labeled lectins (e.g., from Vector labs, usually around 1 : 200 dilution), DAPI*** or phalloidin. Put PCR tubes in dry, foil-wrapped 50 mL Falcon tube (protect from light), place on rocker in 4°C refrigerator, overnight. Lectins that work well in mouse tissues:
   A. peanut agglutinin-rhodamine (PNA) proximal tubules and collecting ducts
   B. lotus tetragonolobus-fluorescein (LTG) proximal tubules
   C. dolichos biflorus-rhodamine (DBA) collecting ducts
   D. lens culinaris agglutin (LCA) GBM, mesangial matrix
8. Next morning **wash** sections in 1× PBS (in 15 mL blue cap tubes), 2 to 4 hours or overnight.

9. Transfer to round base of clean coverslip dishes in preparation for imaging (pre-measure coverslip thickness with micrometer). Keep wet in PBS. To prevent tissue from floating around, you may need to stabilize (not squish! with a cap of cooled agarose or a second coverslip (square or round). Avoid air bubbles!
10. Adjust microscope objective collar to match coverslip thickness.
11. Capture *z*-stacks at 0.4 µm intervals with 60× water-immersion objective.
12. Render 3D image with Voxx or other 3D reconstruction software packages.

Important Points

- *1% is better than 0.1% Triton X-100.
- **Better depth of imaging is achieved with Rhodamine as the label for the fluorescent probe than fluorescein.
- ***DAPI can be added 5 min before imaging (must be washed).

Refractive Index Mismatch

Unfortunately, the imaging of thick sections is often hampered by η mismatch, which can lead to a significant loss of both resolution and signal intensity. The problem becomes serious when one sections more than 10 µm into an aqueous specimen while using a high numerical aperture (NA) oil objective (Hell *et al.*, 1993). The problem can be solved by using objective lenses designed for water immersion together with coverslips with lower η such as CYTOP, which has η = 1.34 (compared to η = 1.33 for water) and 95% transmittance in the range of visible light (developed by Asahi Glass, Yokohama, Japan). Water-immersion objectives that are designed to work without glass coverslips can take advantage of this new material.

Another solution to the RI problem is to mount the specimen in a media that matches both the refractive index of immersion oil and that of a standard coverslip. Mounting media with η = 1.518 can be made from mixtures of glycerol, borate, and potassium iodide (W. McCrone, personal communication). Mounting media with a range of refractive indexes can be bought from R.P. Cargille Labs (NJ) or McCrone Accessories and Components (Westmont, IL).

Table 18.1 lists values of η measured with a Bausch and Lomb, Abbe 3-L refractometer by Dr. M. Wessendorf (University of Minnesota, St. Paul, MN) and Dr. Y. Prakesh (Mayo Clinic, Rochester, MN) for a number of commercially available mounting media. Table 18.2 lists the refractive index of commonly imaged tissues (Biswas and Gupta, 2002).

TABLE 18.1. Refractive Index of Common Mounting Media

Mounting Media	Refractive Index[a]
Gel/mount (Biomeda)	1.3641
Methyl salicylate (Sigma)	1.5409
Dimethyl sulfoxide (Sigma)	1.4836
VectaShield (Vector Labs)	1.4577
DPX (Fluka)	1.5251
50% glycerol/PBS/DABCO	1.4159
Water	1.3381
Cargille index of refraction liquids	1.460–1.700[b]
5% *n*-propyl gallate/0.0025% *p*-phenylene gallate (PPD) dissolved in glycerol	1.4739
0.25% PPD, 0.0025% DABCO, 5% n-propyl gallate dissolved in glycerol	1.4732

[a] Corrected to 20°C.
[b] Can be ordered as a set of liquids with refractive index intervals between each samples as low as 0.002.

TABLE 18.2. Refractive Index of Different Tissue and Organs

Organ/Tissue	Refractive Index
Spleen	1.443 + 0.002
Liver	1.448 + 0.002
Kidney	
Cortex	1.444 + 0.002
Medulla	1.438 + 0.002
Pancreas	1.435 + 0.002
Intestinal wall	1.436 + 0.002
Fat	1.472 + 0.002
Bone	1.556 + 0.002
Cartilage	1.492 + 0.002
Muscle	1.431 + 0.002
Lung	1.342 + 0.002
Gall bladder wall	1.350 + 0.002
Blood (uncoagulated)	
Serum	1.330
Formed elements	1.432 + 0.003
Coagulated blood	1.465 + 0.003
Gray matter	1.395 + 0.002
White matter	1.467 + 0.002
Cerebellum	1.470 + 0.002

From Biswas and Gaupta (2002).

Screening Antibodies on Glutaraldehyde-Fixed Specimens

In our experience, glutaraldehyde fixation best preserves the structural and spatial integrity of the cell. The main difficulty with glutaraldehyde fixation is the frequent loss of epitope antigenicity. However, there may be an alternative to formaldehyde fixation to avoid this problem. It is possible to screen monoclonal antibodies against antigens that had been fixed in glutaraldehyde. Frequently, clones are screened against samples fixed with methanol or formaldehyde, but then they are actually used on glutaraldehyde-fixed specimens. Clearly, this process may produce disappointing results. Better results should be obtained if only those monoclonal antibodies that bind to glutaraldehyde-fixed proteins are used for detailed structural studies.

Microwave Fixation

Microwave heating with and without chemical fixatives has been used for specimen preparation (Thoolen, 1990; Benhamou *et al.*, 1991; Jackson, 1991). The intense heat generated by microwave radiation directly coagulates the proteins and also accelerates chemical fixation. It is not clear what artifacts are created by this fixation method, but some denaturation of protein is to be expected, and there may be problems with reproducibility related to the precise location of the specimen in the chamber, etc. (Giberson and Demaree, 1995). Some investigators have combined chemical fixation with microwave treatment to accelerate the rate of chemical fixation with satisfactory tissue preservation (Jamur *et al.*, 1995; Sawitzky *et al.*, 1996). Clearly, further development of this fixation method might be productive.

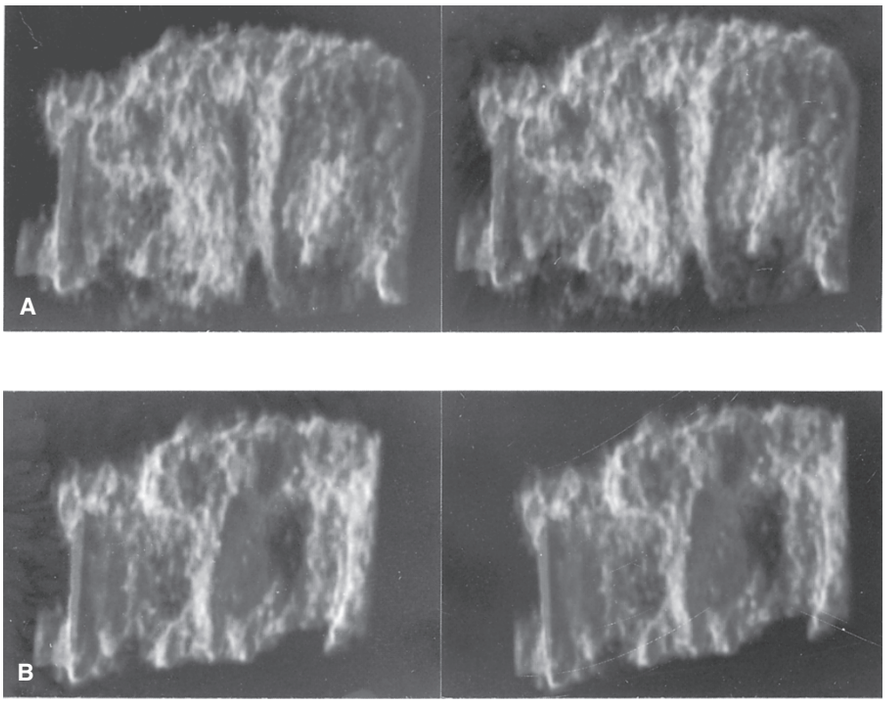

FIGURE 18.5. Stereo image of the actin cytoskeleton in MDCK cells. The cells were labeled with rhodamine phalloidin after fixation with glutaraldehyde. The image was reconstructed using Advanced Visualization Software (Stardent, CA) on a Kubota Pacific workstation. (Photo courtesy of A. Garfinkel and S. Monke.)

A review of the literature shows that the confocal microscope is often used only to produce 2D images and that its ability to generate data sets suitable for 3D reconstruction has been underused. Advances in image-processing and image-analysis techniques now provide the biologist with an array of quantitative 3D measurement tools (Chapters 14 and 15, *this volume*). Figure 18.5 shows such a reconstruction of the actin cytoskeleton of some MDCK cells grown on filter supports in which the images are cut-away views generated by the Advanced Visualization Software program (Stardent, CA) supported by a Kubota Pacific workstation (generously provided by A. Garfinkel and S. Monke, UCLA, Los Angeles, CA). By applying this computer analysis system to this type of data, one can determine the relative intensity values for every voxel in the image, calculate the volume of the actin network in the cell, and determine the volume that demonstrates more than some threshold level of staining as well as displaying a stereo image showing its 3D spatial distribution.

Two-photon microscopy is being used to study tissue morphology due to its ability to penetrate deep into tissue at infrared wavelengths. In our experience we routinely image up to 100 microns into tissue. For our preparations this has been the limit that an image can be acquired. However, we have not performed a rigorous assessment of fixation conditions versus imaging depth. It is possible that optimizing fixation conditions and matching the refractive index of the mounting media may increase the maximal depth of image acquisition. The optimal fluorophores have yet to be determined for imaging deep into tissues and it may be possible to obtain clearer images by measuring the point spread functions at various tissue depths to deconvolved confocal images obtained at greater depths.

It is important to remember that the accuracy of any three-dimensional reconstruction depends on the extent to which the specimen examined in the microscope retained the structural features it had *in vivo*.

CONCLUSION

In molecular cell biology, the microarchitecture of organisms is rapidly becoming a major subject for analysis. For example, the temporal sequence of morphogenetic changes readily observable in embryos makes an excellent system to test the effect of gene deletions, protein overproduction, and changes in transcriptional control on the development of an organism. The ability of the confocal microscope to render accurate, 3D images using immunofluorescence techniques means that it will be a major tool in the analysis of morphogenesis. To ensure that these 3D images carry real 3D information, it is important that whenever possible, structural morphology obtained by various preservation methods should be compared to that observed *in vivo*. In some cases, a compromise will have to be made with respect to fixation methods in order to preserve the antigenicity of a particular protein. These compromises should only be made if the *in vivo* data suggest that the compromise does not affect the relevance of the data. Only vigorous attention to the details of specimen preparation, and in particular, constant comparison between living and prepared specimens, can ensure that an accurate understanding of the 3D structure of the living cell is achieved.

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