Redistribution and differential extraction of soluble proteins in permeabilized cultured cells

Implications for immunofluorescence microscopy

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Summary

Immunofluorescence microscopy is widely used to characterize the cellular distribution of both soluble and structural proteins. Control experiments generally address only the specificity of the antibodies used. The permeabilization/fixed conditions used to prepare cells for antibody application are assumed to preserve faithfully the in vivo distributions of the protein(s) being examined. We systematically tested the extent to which soluble proteins are redistributed into inappropriate locations and are differentially extracted from native locations during the permeabilization and fixation of the cells before antibody application. We separately introduce six soluble FITC-conjugated proteins of different net charges and sizes into living cultured cells. The labeled proteins do not adhere to the external surfaces of living cells and are evenly distributed throughout the cytoplasm with the larger proteins being excluded from the nucleus. The cells are then prepared as if for immunofluorescence using several conditions that encompass many of the methods commonly used for this purpose. Cells permeabilized with 0.1-0.2% Triton X-100 before fixation with 3.7% paraformaldehyde show a striking localization of all but one of the test proteins to the nucleus and/or nucleoli of 60-80% of labeled cells. Punctate cytoplasmic labeling and cytoskeletal-like arrays of labeled protein are also observed. Extraction with 1% detergent prior to fixation removes most but not always all of the exogenous proteins from the cell remnants. Permeabilization of cells with 0.1% detergent after paraformaldehyde fixation leaves a reticular, uneven cytoplasmic distribution of the labeled proteins, and some of the larger proteins are redistributed to the nuclei. Direct fixation/permeabilization with —20°C methanol largely preserves the in vivo distributions of fluorescent proteins with some preferential localization of these proteins to nuclei, nucleoli and the perinuclear region. These results show that misleading apparent localizations of soluble proteins can result from their redistribution and/or differential extraction during the preparation of cells for primary antibody application.

Key words: fluorescence microscopy, immunocytochemistry, immunofluorescence, nucleolus, nucleus, protein localization.

Introduction

Immunofluorescence has been and continues to be an important technique for determining the distribution of proteins within cells. Not only has this methodology been used to examine the distribution of proteins that are functionally and biochemically well characterized, but it has also been used to gain novel information on the location, and hence function, of proteins whose properties are poorly understood.

In the broadest sense, preparation of cells for primary antibody application involves membrane permeabilization to allow entry of the antibodies into the cell and fixation to stabilize cellular structures during subsequent incubations and washes. In practice, three basic approaches are used: (1) gentle detergent extraction followed by mild formaldehyde or paraformaldehyde fixation that preserves antigenic determinants (Osborn and Weber, 1982). This procedure presumably removes some soluble proteins from the cells, leaving structures that can then be visualized against a reduced 'background' signal. (2) Mild formaldehyde fixation prior to detergent permeabilization (Heggeness et al. 1977). This technique is intended to stabilize both soluble and insoluble proteins in their native locations. (3) Concurrent permeabilization and fixation by cold (—10 to —20°C) organic solvents such as methanol (Harris et al. 1980). This procedure precipitates proteins and presumably allows the detection of both soluble and insoluble proteins in their native locations.

Controls for immunolocalization procedures have almost always addressed the specificity of the primary
antibody and the non-specific binding of the fluorescently labeled secondary antibody to cellular components. However, an important, but generally unstated, assumption made in all immunofluorescence studies is that the permeabilization and fixation conditions used to prepare the cells for antibody application faithfully preserve the in vivo distribution of the antigen(s) of interest. This assumption may be appropriate for polymers of proteins, such as actin and tubulin, that are known by independent criteria to form structures that are clearly preserved by the preparation protocols. However, this assumption may or may not be valid for soluble proteins or proteins whose association with cellular structures may be transient or weak.

Assuming that the primary and secondary antibodies are completely specific and that they faithfully display the distribution of the antigen of interest after fixation/extraction, there may be two important interrelated sources of error in localizing soluble or partially soluble proteins. First, permeabilization before fixation could allow such proteins to be redistributed into locations in which they are not present in vivo. Fixation of proteins in these locations would inevitably yield misleading results. Second, loss of proteins from cells during permeabilization and fixation could lead to the differential extraction of soluble proteins from compartments or structures in which they are normally present. Even if the soluble proteins do not bind to any cellular structures, they would appear to be preferentially localized to compartments or volumes of cytoplasm from which they are least easily extracted. Furthermore, both sources of error could operate together. These errors could also occur even when cells are lightly fixed with monofunctional fixatives, such as formaldehyde, before permeabilization. Unless the fixative completely immobilizes all soluble proteins, redistribution and differential extraction could lead to artifactual distributions of the antigens.

In principle, the best way to determine the in vivo distribution of soluble proteins is by fluorescent analog cytochemistry of living cells (Wang, 1989). However, relatively large quantities of a protein must be purified in native form, and the derivatization of the protein with a chromophore must demonstrably not alter its functional properties. Thus, it is not surprising that fluorescent analog cytochemistry of living cells has been used in relatively few cases.

Although the potential for artifacts due to the redistribution and differential extraction of soluble proteins has not been systematically examined, there have been indications that these errors can be a practical problem. One of the clearest examples comes from a comparison between the in vivo distribution of fluorescently labeled gelsolin and its cellular localization as determined by immunofluorescence (Cooper et al. 1988). This study revealed a clear disparity in the localization of gelsolin as determined by the two methods. Other studies report that protein distributions can vary with the preparation technique used. For example, the distributions of calmodulin (Nielsen et al. 1987) and of a nuclear antigen (Hoffman and Mullins, 1990) are different depending upon whether the cells are permeabilized before or after fixation. Also, Pettijohn et al. (1984) clearly show that the NuMA antigen can exchange between cellular structures during the preparation of cells and isolated chromosomes for immunofluorescence. Furthermore, the reported intracellular distribution of the same protein can differ from laboratory to laboratory. Kinesin, which is largely soluble in vitro, has been reported to be: evenly distributed in the cytoplasm (Hollenbeck, 1989); on microtubules, spindles, centrosomes, primary cilia and endoplasmic reticulum (Neighbors et al. 1988; Scholey et al. 1985; Terasaki, 1990); or associated exclusively with cytoplasmic vesicles (Pfister et al. 1989).

We have systematically investigated the extent to which redistribution and differential extraction can produce artifactual distributions of soluble proteins during the preparation of cells for immunofluorescence. Our approach has been to bead-load several types of cultured cells with fluorescently labeled soluble proteins of various molecular weights and net charges. We then compared the in vivo distribution of each soluble protein with its distribution after preparing the cells as if for immunofluorescence microscopy using several preparation conditions that encompass many of the commonly used methods.

Materials and methods

Living material

PtK1 and CHO cells were maintained at 37°C in L-15 (Leibovitz) medium (Sigma, St Louis, MO) buffered with 10 mM Hepes and supplemented with 10% fetal bovine serum (Hazelton Biologies, Inc., Lenexa, KS). HeLa and 3T3 cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum in a 5% CO2 atmosphere. All cells were grown on 22 mm × 22 mm glass coverslips in 6-well tissue culture plates and used at subconfluent densities.

Fluorescein-labeled protein was introduced into cells using the bead-loading technique of McNeil and Warder (1987). Glass beads (150-212 μm, G-9018, Sigma) were treated with 4 M NaOH for 1 h, washed with distilled water until the pH of the water remained constant, then dried at 70°C overnight. Coverslips with cells were rinsed with PBS then flooded with 35 μl of labeled protein. The cells were then covered with a monolayer of washed glass beads and agitated vigorously by hand for approximately 10 s. The glass beads were washed from the cells by dipping the coverslips into PBS. The cells were allowed to recover in culture medium at 37°C for 1 h, before extraction and fixation.

Protein labeling

Ovalbumin (A-2512, Grade III), bovine serum albumin (BSA) (A-4503, Fraction V), α-lactalbumin B (L-8005), globin (G-0981), L-lactic dehydrogenase (LDH) (L-1254, Type XI), and cytochrome c (C-2506, Type III) (all from Sigma Chemical Co., St Louis, MO) were labeled with fluorescein-5-isothiocyanate (FITC) (F-143, Molecular Probes Inc., Eugene, OR) using a modification of the procedures of Harlow and Lane (1988). Three mg of protein were dissolved in 1 ml of 0.1 M sodium carbonate, pH 9.0. A solution of 10 mg/ml FITC in DMSO (dimethyl sulfoxide) was
slowly added to the protein solution in samples (5 µl each) up to an approximately 20:1 molar ratio of dye to protein. Samples were incubated in darkness at 4°C for 18 h. The labeling reaction was quenched by adding NH₄Cl to a concentration of 50 mM. Unbound dye was separated from labeled protein by gel filtration over Sephadex G-25M columns (PD-10, Pharmacia, Uppsala, Sweden) using phosphate buffered saline (PBS) for column equilibration and elution of the protein. Labeled protein in PBS was stored in amber tubes at 4°C or frozen in samples at −80°C. Protein concentrations were determined using a modified Lowry micro-assay (Peterson, 1977) with BSA as the protein standard. Fluorescein/protein ratios were calculated from absorbance values at 495 nm obtained with a Beckman DU-50 Spectrophotometer, and protein concentration data. Protein samples were diluted into 50 mM Tris-HCl, pH 8.0, before measurement of the absorbance values. A molar absorption coefficient value for fluorescein of ε=65,000 M⁻¹ cm⁻¹ was used in the calculations (Molecular Probes, Inc., Eugene, OR).

**Gel electrophoresis**

Fluorescein-labeled proteins were compared with their unlabeled precursors on 10% to 20% gradient SDS/polyacrylamide gels (Laemmli, 1970). Before fixation and staining of the gels, fluorescein-labeled proteins were visualized on an ultraviolet transilluminator (emission wavelength 302 nm, Model TM-36, UVP, Inc., San Gabriel, CA). Total protein was stained in the gels with Coomassie blue.

**Extraction and fixation of cells**

Before fixation procedures were carried out, coverslips with attached cells were washed twice with PBS for 30 s each at 25°C.

**Extraction followed by fixation**

Cells were extracted for 10 min with 0.1 M Pipes, 10 mM EGTA, 5 mM MgCl₂, pH 6.9 (PEM buffer), at 37°C containing Triton X-100 or Nonidet P-40 (Sigma, St. Louis, MO) at concentrations of 0.1, 0.2, 0.5 or 1%. Also, high purity Triton X-100 (Surfact-Amps X-100, Pierce Chemical Co., Rockford, IL) was used in some experiments at the same concentrations. Detergent-extracted cells were fixed for 30 min at 25°C and then permeabilized with 0.1% Triton X-100 or 3.7% formaldehyde (Scientific Products, McGraw Park, IL) at concentrations of 0.1, 0.2, 0.5 or 1%. Also, high purity Triton X-100 (Surfact-Amps X-100, Pierce Chemical Co., Rockford, IL) was used in some experiments at the same concentrations. Detergent-extracted cells were fixed for 30 min at 25°C in extraction medium containing either 3.7% paraformaldehyde (Polysciences, Inc., Warrington, PA) or 3.7% formaldehyde (Scientific Products, Mcgraw Park, IL).

**Fixation before detergent permeabilization**

Cells were fixed with 3.7% paraformaldehyde in PEM buffer for 30 min at 25°C and then permeabilized with 0.1% Triton X-100 in PEM for 10 min at 25°C.

**Solvent fixation/permeabilization**

Cells were directly fixed and permeabilized in 90% methanol, 50 mM EGTA, pH 6.0, for 5 min at −20°C (Osborn and Weber, 1982).

**Fixation followed by solvent extraction**

Cells were fixed with 3.7% paraformaldehyde in PEM for 30 min at 25°C and then permeabilized in either absolute acetone at −20°C or 90% methanol, 50 mM EGTA, pH 6.0, at −20°C for 5 min.

After the fixation/permeabilization regime, the coverslips were rinsed with PBS and mounted onto slides with a polyvinyl alcohol mounting medium (Osborn and Weber, 1982) containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane to reduce photobleaching of the fluorescein (Johnson et al. 1982).

**BSA immuno-fluorescence**

Following fixation and permeabilization (as above), the cells were blocked with 1% ovalbumin (Calbiochem, San Diego, CA) in PBS for 10 min. Cells were then exposed to a 1:500 dilution of mouse monoclonal anti-bovine serum albumin (Sigma, St. Louis, MO) for 30 min followed by treatment with a 1:100 dilution of fluorescein-labeled sheep anti-mouse IgG (Sigma, St. Louis, MO) for 10 min. Antibodies were diluted with PBS containing 1% ovalbumin. Coverslips were mounted as described above.

**Fluorescence microscopy**

Slides were observed with a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) equipped for both phase-contrast and epifluorescence microscopy. A narrow bandpass filter set (Carl Zeiss, Inc., Thornwood, NY) was used for fluorescein observations. Photographs were taken with a Zeiss MCI100 camera (automatic exposure) on Kodak T-Max 400 film and developed in Kodak D-76 developer (Eastman Kodak, Rochester, NY).

To obtain the quantitative data shown in Table 2 (below), we scored only cells that showed fluorescent labeling. Since any given cell could be scored in more than one category, the sum of the percentages for any one protein may be greater than 100%.

**Results**

We tested the cellular distribution of soluble proteins after preparing cells as if for immuno-fluorescence by several commonly used approaches: detergent extraction followed by formaldehyde fixation, formaldehyde fixation followed by detergent permeabilization, and direct permeabilization/fixedation with cold methanol. Given the extremely numerous slight modifications of these basic themes used by various workers, we did not attempt to duplicate precisely the conditions used in all previous studies. Instead, we used conditions that encompassed the particular methods that have been commonly used.

**Protein labeling and characterization**

Table 1 lists the properties of the protein reagents that were individually bead-loaded into cells. Protein solutions were applied extracellularly at the concentrations indicated. The electrophoretic patterns of the FITC-labeled proteins are shown in Fig. 1A. The fluorescence of the labeled ovalbumin (lane 1, asterisk) is barely visible on the gel due to the low level of FITC binding to this protein (F/P=0.47). The minor bands visible in some lanes are probably due to contaminants due to serum albumin impurity in the sample.
Table 1. Fluorescein-labeled protein samples

<table>
<thead>
<tr>
<th>Protein</th>
<th>$M_r$ ($\times 10^{-3}$) (unmodified protein)</th>
<th>Isoelectric point (unmodified protein)</th>
<th>Concentration (mg/ml)</th>
<th>Fluorescein/protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>45</td>
<td>4.6</td>
<td>0.97</td>
<td>0.47</td>
</tr>
<tr>
<td>BSA</td>
<td>66</td>
<td>4.9</td>
<td>0.91</td>
<td>2.23</td>
</tr>
<tr>
<td>$\beta$-Lactoglobulin B</td>
<td>18.4</td>
<td>5.1</td>
<td>0.62</td>
<td>1.14</td>
</tr>
<tr>
<td>Globin</td>
<td>62</td>
<td>8.5</td>
<td>1.05</td>
<td>1.49</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td></td>
<td>2α chains (15.0)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>2β chains (15.9)</td>
<td></td>
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</tr>
<tr>
<td>Cytochrome c</td>
<td></td>
<td>140</td>
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<td></td>
</tr>
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</table>

Fig. 1. SDS-polyacrylamide gel electrophoresis of FITC-labeled and native proteins. (A) FITC-conjugated proteins viewed with an ultraviolet transilluminator ($\lambda=302$ nm). Lanes 1-6, ovalbumin, BSA, $\beta$-lactoglobulin B, globin, LDH and cytochrome c, respectively. Asterisk marks the band of ovalbumin, which is barely visible. (B) Coomassie blue-stained gel of FITC-labeled protein and native protein run in adjacent lanes. Lanes 1-2, ovalbumin; 3-4, BSA; 5-6, $\beta$-lactoglobulin B; 7-8, globin; 9-10, LDH; 11-12, cytochrome c. Relative molecular mass markers ($\times 10^{-3}$) are indicated to the left of A. Five $\mu$g protein/lane.

Distributions of labeled protein in living cells

During the bead-loading procedure, the living cells are exposed to labeled proteins for 0.5-1 min. To test for possible binding of the labeled proteins to external cell surfaces, living PtK$_1$ cells were exposed to the fluorescent proteins for 10-30 min and then rinsed with PBS prior to observation in vivo on the fluorescence microscope. Fig. 2A-A' shows phase-contrast and fluorescence micrographs of a group of cells after a 10 min exposure to labeled cytochrome c. Fig. 2B-B' shows another field of cells following exposure to FITC-globin for 20 min. For all tests, either no fluorescence was observed or a very low level of fluorescence was seen. The low level of fluorescence shown here has been accentuated by the long photographic exposures due to the automatic exposure control of the camera. These observations indicate that live PtK$_1$ cells do not bind significant amounts of FITC-labeled proteins to their external surfaces during the 0.5-1 min duration of the bead-loading procedure. After greater than 30 min exposure to labeled proteins in the medium, some cells contained punctate fluorescence in the perinuclear region, suggesting pinocytotic uptake (data not shown).

Following bead-loading, individual cells showed varying levels of protein incorporation (Fig. 2C-C'). The variable quantity of protein incorporated after bead-loading occurred for all proteins in all the cell types that we examined and is consistent with the findings of McNeil and Warder (1987).

Fig. 3 A-F shows representative examples of the in vivo distributions of the 6 FITC-labeled proteins in living cells. Proteins with $M_r$ values greater than ~50,000 were evenly distributed throughout the cytoplasm but excluded from the nucleus. Proteins with $M_r$ values less than ~50,000 were distributed throughout both the cytoplasm and the nucleus. The in vivo distribution of globin sometimes showed two types of localization (Table 2; Fig. 3D inset). The nuclear localization of FITC-globin may reflect the dissociation of some globin molecules into subunits that are smaller than 50,000 $M_r$. In no case did we observe preferential localization of labeled proteins into linear cytoplasmic arrays, perinuclear spots or nucleoli (Table 2).

Distributions of labeled protein in cells extracted with detergent before fixation

We extracted interphase PtK$_1$ cells loaded with FITC-labeled proteins with Triton X-100 or Nonidet P-40 before fixation with either formaldehyde or paraformaldehyde. We used a range of detergent concentrations that include those found in many published studies: 0.1% (Bonifacino et al. 1985; Goldenthal et al. 1985; Hoffman and Mullins, 1990), 0.2% (Osborn and Weber, 1982; Vandré et al. 1984; Vandré et al. 1986) and 1% (Bályicz and Schatten, 1983; Hollenbeck, 1989). As a standard, we used a 10 min extraction time. Published extraction times range from 1 to 60 min, and our control experiments using longer (20-30 min) or shorter (2-5 min) extraction times revealed that the results shown below are not qualitatively sensitive to variations in extraction duration (data not shown).

Extraction with 0.1% detergent prior to fixation

Fluorescent protein distributions in interphase PtK$_1$
Fig. 2. (A, A') Phase-contrast and fluorescence micrographs of living PtK1 cells treated with 0.91 mg/ml FITC-cytochrome c for 10 min without bead-loading. (B, B') Phase-contrast and fluorescence micrographs of living PtK1 cells treated with 1.05 mg/ml FITC-globin for 20 min without bead-loading. Note low fluorescence in A' and B', indicating a lack of binding to the cell surfaces. (C, C') Phase-contrast and fluorescence micrographs of a field of living PtK1 cells bead-loaded with FITC-globin. Cells were exposed to external labeled protein for <1 min. Variations in the levels of fluorescence intensity indicate different amounts of protein loaded into the cells. 10 μm per scale division (black bars in all Figs).

Fig. 3. FTTC-protein distributions in living PtK1 cells. (A-F) Ovalbumin, BSA, β-lactoglobulin B, globin, LDH and cytochrome c, respectively. Proteins are distributed evenly throughout the cells with proteins of Mr greater than 50,000 excluded from the nuclei (B, D, E). In some cases, globin also exhibits nuclear fluorescence (D, inset). 10 μm per scale division.

Fig. 4. FITC-protein distributions in PtK1 cells following a 10 min extraction with 0.1% Triton-X 100 and 30 min fixation with 3.7% paraformaldehyde. (A-F) Ovalbumin, BSA, β-lactoglobulin B, globin, LDH and cytochrome c, respectively. All proteins except cytochrome c (F) show retention within the cells. The greatest fluorescence intensities are seen in the nuclei and nucleoli. A low percentage of cells (2%) loaded with FITC-BSA (B) show a pattern of fluorescence in the cytoplasm indicative of cytoskeletal association. Arrows in C, D, and E indicate juxtanuclear localizations. 10 μm per scale division.

cells that are extracted for 10 min with 0.1% Triton X-100 or Nonidet P-40 prior to fixation are shown in Fig. 4 A-F. For cells loaded with cytochrome c, the smallest of the test proteins, such extraction removes essentially all the labeled protein from the nucleus and cytoplasm (Fig. 4F and Table 2). For cells loaded with the other test proteins, extraction removes much, but not all, of the fluorescent probes from the cytoplasm leaving diffuse, reticulate or linear cytoplasmic arrays of fluorescent material in approximately half of the loaded cells (Table 2). In 2% of the cells labeled with BSA, the protein appears to be associated with linear cytoskeletal
Table 2. Frequency of localization patterns (%)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein</th>
<th>Cytoplasmic</th>
<th>Nuclear</th>
<th>Nucleolar</th>
<th>Other</th>
<th>Cells scored (n)</th>
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<td></td>
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<tr>
<td>Ovalbumin</td>
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<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>BSA</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>β-Lactoglobulin B</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>61</td>
</tr>
<tr>
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<td>58</td>
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<td>79</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>43</td>
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<tr>
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<td>100</td>
<td>100</td>
<td>0</td>
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<td>55</td>
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<tr>
<td><strong>Detergent extraction before fixation</strong></td>
<td></td>
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<td>8</td>
<td>2*</td>
<td>188</td>
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<td>60</td>
<td>5</td>
<td>2*</td>
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<td>6†</td>
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<td>3</td>
<td>7†</td>
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<td>30†</td>
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*Linear cytoplasmic elements; †juxtanuclear spots; ‡primary cilia.

arrays (Fig. 4B). Also, small percentages of cells show localization of β-lactoglobulin B, globin and LDH to a single focus next to the nucleus (Table 2 and Fig. 4C-E, arrows).

With the exception of cytochrome c, all labeled proteins are strikingly localized in the nucleus and/or nucleoli of 68-80% of the loaded cells (Fig. 4A-E and Table 2). BSA, however, generally was not redistributed to the nucleoli (Fig. 4B and Table 2), and preferential localization of other proteins, such as globin (Fig. 4D), to nucleoli was observed at a lower frequency than for nuclear localization (Table 2).

We established the percentage of label retention in various cellular domains only in cells that showed at least some fluorescence (Table 2). Thus, these data show the relative frequency of the different categories of label distribution. Cells loaded with small amounts of labeled protein and cells from which the label had been completely extracted are not scored. In this regard, however, we did not observe any obvious, systematic reduction in the total percentage of cells in a preparation that were labeled after detergent extraction, except where indicated in Table 2.

The fluorescence in the detergent-extracted cells shown here and in other experiments is not caused by fluorescent impurities in either the fixative or detergent. Control extractions/fixations conducted on cells that were not loaded with FITC-labeled protein showed neither background fluorescence nor localized fluorescence. Furthermore, we found no difference in structural preservation and/or fluorescence distribution when reagent grade Triton X-100 or formaldehyde was
used and no difference in fluorescence distribution if Nonidet P-40 was used in place of Triton X-100.

**Extraction with 0.2% detergent prior to fixation**
Fluorescent protein distributions in interphase PtK₁ cells that are extracted for 10 min with 0.2% Triton X-100 or NP-40 prior to fixation are shown in Fig. 5 A-F. For cells loaded with cytochrome c and ovalbumin, this extraction removes essentially all the the labeled protein from the nucleus and cytoplasm (Fig. 5A, 5F and Table 2). For cells loaded with the other test proteins, labeled proteins were localized to nuclei and nucleoli (Fig. 5B-E). There is a low but finite incidence of labeled protein localization to linear arrays in the cytoplasm reminiscent of microtubules (seen only with BSA) and to single juxtanuclear spots (Fig. 5B). In 4% of the cells loaded with FITC-globin, we observed label localization to a single linear element on each cell, which may be the primary cilium (Fig. 5D, lower inset). The higher percentage of cells that show nucleolar localization of globin relative to corresponding cells extracted with 0.1% detergent may reflect the higher contrast of nucleolar label against a reduced background of nuclear label.

**Extraction with 1% detergent prior to fixation**
Fluorescent protein distributions in interphase PtK₁ cells that are extracted for 10 min with 1% Triton X-100 prior to fixation are shown in Fig. 6 A-F. In general, the higher detergent concentration leads to a noticeably greater extraction of all test proteins. Essentially all ovalbumin, BSA, LDH and cytochrome c are removed from both cytoplasmic and nuclear domains (Fig. 6A,B,E,F and Table 2). Only cells loaded with β-lactoglobulin B and globin showed retention of some label in cytoplasmic, nuclear and nucleolar domains (Fig. 6C-D). These labeled proteins were most frequently retained in the nucleoli (Table 2).

**Fixation before permeabilization**
We fixed living PtK₁ cells for 30 min with 3.7% paraformaldehyde, then extracted them with 0.1% Triton X-100 according to the methods outlined by Cooper et al. (1988) and Hoffmann and Mullins (1990).

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Fig. 5. FITC-protein distributions in PtK₁ cells following a 10 min extraction with 0.2% Triton-X 100 and a 30 min fixation with 3.7% paraformaldehyde. (A-F) Ovalbumin, BSA, β-lactoglobulin B, globin, LDH and cytochrome c, respectively. Ovalbumin (A) and cytochrome c (F) are completely removed from the cells. The remainder of proteins show strong localization in the nuclei and/or nucleoli. Approximately 2% of cells loaded with FITC-BSA (B) show possible cytoskeletal association. Cells loaded with FITC-globin (D) show a variety of localizations including nuclei and centrosomes (upper inset) and primary cilia (lower inset). Arrows in B and D (upper inset) indicate juxtanuclear localization. 10 μm per scale division.

Fig. 6. FITC-protein distributions in PtK₁ cells following a 10 min extraction with 1% Triton X-100 and 30 min fixation with 3.7% paraformaldehyde. (A-F) Ovalbumin, BSA, β-lactoglobulin B, globin, LDH and cytochrome c, respectively. All proteins except β-lactoglobulin B (C) and globin (D) are removed from the cells. The strongest fluorescent signals are seen exclusively in the nucleoli with some cells showing retention of label in the nucleus as well (D, inset). 10 μm per scale division.
Fig. 7. FITC-protein distributions in PtK₁ cells fixed for 30 min with 3.7% paraformaldehyde then permeabilized with 0.1% Triton X-100 for 10 min. (A-F) Ovalbumin, BSA, β-lactoglobulin B, globin, LDH and cytochrome c, respectively. All proteins show a reticulate cytoplasmic distribution. Cells loaded with FITC-BSA, globin and LDH display nuclear fluorescence in contrast to the in vivo exclusion of these proteins from the nuclei. The cell-to-cell variability of this technique is shown in D. Possible association of label with the interphase centrosome was frequently seen with this technique (arrows, A, C, D and F). 10 μm per scale division.

For FITC-conjugated ovalbumin, β-lactoglobulin B and cytochrome c (Fig. 7A, C and F, respectively) fluorescence distribution after fixation and extraction resembled the in vivo distribution, with two exceptions: the cytoplasmic domains had a coarser, more reticulate appearance and 4-9% of the labeled cells showed a single bright spot adjacent to the nucleus (7A, C and F, arrows and Table 2). The position and number of these loci were confirmed by focusing through the cell.

The cytoplasmic distribution of FITC-conjugated BSA, globin and LDH also appeared to be more reticulate than in vivo (Fig. 7B, D and E, respectively) and was sometimes noticeably punctate. Six to 30% of the labeled cells showed a single spot next to the nucleus in the expected location of the centrosome. In 35 and 49% of the cells loaded with BSA and LDH, respectively, we observe a striking relocalization of labeled proteins to the nucleus (Table 2). Labeled BSA relocalized to and was retained in the nuclear matrix but not in the nucleoli (Fig. 7B).

Permeabilization of membranes following chemical fixation can also be accomplished by using organic solvents in place of detergents (Osborn and Weber, 1982; Baron and Salisbury, 1988). We conducted seven separate trials in which we permeabilized cells with either methanol or acetone after fixation. In all experiments, the remaining fluorescent signal was low and consequently we were unable to determine the distribution of remaining labeled proteins, if any, within the cell remnants (data not shown).

Direct fixation/permeabilization with cold methanol

Cells were loaded with FITC-protein and then directly fixed/permeabilized with 90% methanol containing 50 mM EGTA at -20°C (Osborn and Weber, 1982). In general, the distributions of labeled proteins approximated, but did not duplicate, the corresponding protein distributions in vivo (Fig. 8A-F vs Fig. 3A-F). The cytoplasmic distribution of fluorescence exhibited a fibrous, reticulate texture not observed in vivo and the overall fluorescent intensity of the population of fixed cells was significantly lower than in vivo. Larger proteins for the most part remained excluded from the nucleus; however, cytoplasmic fluorescence above and

Fig. 8. FITC-protein distributions in PtK₁ cells after fixation for 5 min in 90% methanol, 50 mM EGTA at -20°C. (A-F) Ovalbumin, BSA, β-lactoglobulin B, globin, LDH and cytochrome c, respectively. All proteins show a fibrous textured fluorescence in the cytoplasm and stronger fluorescence at the periphery of the nucleus. Globin (D) and cytochrome c (F) also show fluorescence in the nucleoli. The overall level of fluorescence for all proteins is lower than that seen in vivo. 10 μm per scale division.
below the nucleus sometimes made this determination difficult (Fig. 8B and E). In many cells, the fluorescence was greater in the region surrounding the nucleus than in the peripheral cytoplasm (Fig. 8C, D and E). This may be due to the greater cytoplasmic thickness in these regions coupled with the partial extraction of the test proteins from the cytoplasm. In addition, the proteins β-lactoglobulin B, globin and cytochrome c appear to be preferentially retained in the nucleoli (Table 2).

**Localization of underivitized BSA**

We tested the formal possibility that the distributions of labeled proteins shown in Figs 4-8 could be due partially or totally to peculiar properties of these proteins caused by derivitization with FITC. Thus, we bead-loaded unmodified BSA (0.91 mg/ml in PBS) into PtK1 cells and processed them in the same ways as the cells loaded with FITC-proteins. We then used indirect immunofluorescence to determine the distribution of the unmodified BSA. Fig. 9A, B and C, respectively, show cells that were extracted with 0.1%, 0.2% and 1% detergent prior to formaldehyde fixation. The distributions of unmodified BSA match those of the FITC-BSA (see Figs 4B, 5B and 6B). Fig. 9D shows the distribution of unlabeled BSA in a cell that was fixed with paraformaldehyde before extraction with 0.1% detergent (compare with the FITC-BSA distribution shown in Fig. 7B). The distribution of unlabeled BSA in a cell that was fixed/permeabilized with cold methanol is shown in Fig. 9E (compare with the FITC-BSA distribution shown in Fig. 8B). The higher cytoplasmic fluorescence of cells prepared by indirect immunofluorescence compared to those loaded with FITC-BSA (Fig. 9E vs Fig. 8B) may represent signal amplification due to the binding of multiple primary and secondary antibodies.

**Protein localizations in different cell types**

To determine if the results shown above are unique to PtK1 cells, parallel experiments were conducted with CHO, 3T3 and HeLa cells bead-loaded with FITC-BSA as the test protein. In living cells the FITC-BSA is uniformly distributed throughout the cytoplasm and is excluded from the nucleus in all cell types (Fig. 10 A-D).

The distribution of FITC-BSA in cells extracted with 0.1% detergent and then fixed with formaldehyde is shown in Fig. 10 E-H. In PtK1 cells, labeled BSA is seen primarily in the nucleus (excluding the nucleoli) with some retention in the cytoplasm. For CHO cells, this extraction removes almost all of the tagged protein; only a very weak cytoplasmic signal can be detected in some cells (Fig. 10 F). In 3T3 cells the FITC-BSA is seen in the nucleus and possibly the nucleoli; labeled protein is generally completely extracted from the cytoplasm (Fig. 10 G). HeLa cells generally show weak cytoplasmic fluorescence with some of the labeled protein in the nucleus (Fig. 10 H).

The distribution of FITC-BSA in cells extracted with 1% detergent and then fixed with formaldehyde, is shown in Fig. 10 I-L. With the exception of label in the nuclei of a low percentage of the HeLa cells, this regime extracts the labeled probe from all cell types.

The localization of FITC-BSA in cells fixed with paraformaldehyde before permeabilization with 0.1% Triton X-100 is shown in Fig. 10 M-P. For all cell types, the cytoplasmic distribution of label has a coarse, fibrous appearance with areas devoid of label. This is in clear contrast to the label distribution in living cells where the loaded protein is uniformly dispersed throughout the cytoplasm. In CHO cells there is a striking relocalization of FITC-BSA to the nucleus but not the nucleoli (Fig. 10 N). This same relocalization
occurs in PtK₁ cells as well, but to a lesser extent (Fig. 10 M).

The distribution of FITC-BSA after direct fixation/permeabilization with methanol at -20°C is shown in Fig. 10 Q-T. For all cell types the cytoplasmic distribution of the labeled protein has a coarse fibrous texture and an overall low fluorescence intensity. The label is not redistributed to the nuclei of PtK₁ and HeLa cells but is to a limited extent in CHO and 3T3 cells (Fig. 10 R and S). In addition, this technique enucleates most of the 3T3 cells (data not shown).

Global redistribution of labeled protein
We often observed that low levels of fluorescence were present in the cytoplasm of all cells on the coverslip for all of the techniques and for all cell lines used in this study. This is not due to uniform loading of cells, as...
This protocol is often used to remove soluble proteins to cellular components. Any redistribution of soluble proteins is most clearly demonstrated by our *in vivo* observations (Fig. 2C'), or to autofluorescence of the detergent or fixative. This global fluorescence suggests that label extracted from loaded cells diffuses to and becomes trapped in other cell remnants during extraction and fixation.

**Discussion**

Immunofluorescence microscopy has been used to determine the cellular localization of proteins in order to gain insight into their function. This approach has been particularly important for studies on proteins that are poorly understood. However, any conclusion about the localization of a protein, and hence its presumed function, critically depends upon the faithful preservation of its *in vivo* distribution. Any redistribution or differential extraction of the antigen of interest during the preparation of the cells for immunofluorescence could suggest interactions or functions that are incorrect.

To provide the clearest possible test for the redistribution and differential extraction of proteins, we separately introduced six FITC-labeled proteins into cultured cells and directly compared their *in vivo* localizations with their distributions after permeabilization and fixation as if for immunofluorescence. Direct labeling of the proteins allowed us to avoid questions about antibody specificity and non-specific binding of secondary antibodies to cellular components. In addition, four of the six test proteins should not normally be present in these cells; this reduces the possibility of specific or physiologically relevant binding of the test proteins to cellular components.

**Detergent extraction before fixation**

This protocol is often used to remove soluble proteins and the unpolymerized pools of cytoskeletal proteins prior to fixation and application of the primary antibody. Given that we loaded the cells with soluble proteins that should not have physiologically relevant interactions with cellular components, we could have expected the test proteins to have been completely removed from the cell remnants, or reduced but remaining in the *in vivo* locations. Our results show, on the contrary, that extraction of cells with nonionic detergents before fixation with monofunctional aldehydes can lead to both the redistribution and differential extraction of soluble proteins producing apparent localizations that are entirely artificial. The redistribution of soluble proteins is most clearly demonstrated in cells loaded with the higher *M* subunits, such as BSA, and extracted with 0.1% or 0.2% detergent. In living cells, these proteins are uniformly distributed in the cytoplasm but excluded from the nucleus. After extraction and fixation, they are strikingly localized in the nucleus and/or nucleoli in the majority of the labeled cells. In addition, extraction of the labeled proteins from the cytoplasm was not complete or uniform; we observed label in cytoskeletal-like arrays and in a single spot next to the nucleus, which could be the remnant of the interphase centrosome.

These results are not due to the fluorescein moiety, which conceivably might impart unusual properties to the test probes that would cause them to be distributed in a qualitatively different fashion than native proteins. Indirect immunofluorescence of cells loaded with native BSA showed in all cases that the distribution of native BSA was the same as that of derivatized BSA. In addition, control experiments show that the distribution of fluorescence could not be due to the fixatives or detergents themselves or to impurities in these reagents.

We found that the extent of the extraction of labeled proteins was a function of detergent concentration, protein size, protein charge, cellular structure and cell type. Cytochrome *c*, a relatively small and basic protein, is essentially completely extracted from both the cytoplasmic and nuclear compartments by all detergent extractions used. For larger proteins, increasing detergent concentration reduced but did not always eliminate the residual labeled protein found in both the nucleus and cytoplasm.

Our comparison of the distribution of labeled BSA in different cell types shows that the extent of extraction of this protein can also vary between cell types. For example, at low detergent concentrations (0.1%) the label is noticeably more extracted from CHO and HeLa cells than from PtK1 and 3T3 cells. Extraction with 1% detergent, however, removes most of the labeled BSA from all cell types.

**Fixation before permeabilization**

In principle, fixation with paraformaldehyde prior to permeabilization should faithfully preserve the *in vivo* distribution of all proteins by fixing them in place before the cell is permeabilized with detergent. However, our results reveal that redistribution and differential extraction of soluble proteins can occur in cells prepared by this technique. We found that the cytoplasmic distribution of the labeled proteins had a coarse, punctate and sometimes reticulate appearance that differed from the uniform *in vivo* distribution. Depending on the particular protein, between 4 and 30% of the labeled cells exhibited a single fluorescent spot next to the nucleus, which may represent preferential retention of probe in the remnant of the centrosome. Importantly, for greater than 35% of the cells loaded with labeled BSA, globin and LDH, we observe a relocalization of labeled proteins to the nucleus.

We also found that the extent of the discrepancy between the *in vivo* and post-preparation distributions of a soluble protein can vary between cell types. The distribution of labeled BSA in fixed/permeabilized 3T3 and HeLa cells showed the closest resemblance to the *in vivo* distribution. However, in PtK1 and CHO cells, the cytoplasmic distribution of labeled BSA was noticeably coarser and there was clear evidence for redistribution of the probe into the nucleus where it is not found *in vivo*.

**Direct fixation/permeabilization with cold methanol**

This protocol is commonly assumed to precipitate...
completely both structural and soluble proteins in place without loss, thereby faithfully representing the in vivo distribution. Our results show that the distributions of labeled proteins did not duplicate the corresponding protein distributions in vivo. The cytoplasmic distribution of fluorescence exhibited a fibrous, reticulate texture not observed in living cells and the overall fluorescence intensity of the populations of fixed cells was lower than the in vivo fluorescence. This reduction in fluorescence intensity may be due to the partial extraction of proteins (Fujiwara and Pollard, 1980). A practical consequence of this partial extraction, for some cell types, is that the antigen of interest can appear to be preferentially localized around the nucleus and not uniformly distributed throughout the cytoplasm as it was in vivo. In addition, for some proteins such as labeled cytochrome c and globin, this protocol leads to the apparent preferential retention of the proteins in the nucleoli. There is also a low but finite redistribution of higher $M_r$ proteins into the nuclear and nucleolar compartments.

**Conclusions**

Our results demonstrate that none of the permeabilization/fixation methods we tested completely preserves the in vivo distribution of soluble proteins. These methods commonly used to prepare cells for immunofluorescence can lead to the redistribution and differential extraction of soluble proteins before antibody application, thereby producing misleading patterns for the localization of such proteins. If indirect immunofluorescence is subsequently used, possible signal amplification for artificially distributed proteins could make their localizations appear striking and, therefore, subjectively real. Since these artifacts will be superimposed upon the real and physiologically relevant distributions of the proteins of interest, the interpretation of immunofluorescent images is bound to be difficult. Unless the problems of redistribution and differential extraction can be demonstrably excluded, common sense must be used in matching the particular protocol used to prepare cells for immunofluorescence with the expected properties of the protein being examined and the sort of information being sought. Also, images should be evaluated with the realization that partial and differential extraction of the antigen are definite possibilities.

While use of the bifunctional cross-linking fixative glutaraldehyde should produce superior antigen immobilization, this fixative is not commonly used for immunofluorescence, due to non-specific background fluorescence and/or loss of antigenicity (Nakane, 1975; Cande et al. 1977; Weber et al. 1978). Our attempts to demonstrate the fixation of soluble proteins with glutaraldehyde resulted in very high levels of overall fluorescence even after treatment of the cells with sodium borohydride (Weber et al. 1978). Nonetheless, DeBrabender et al. (1977) used glutaraldehyde fixation to show effectively the localization of the soluble pool of tubulin in cells treated with colchicine or vinblastine. However, they localized the protein within the cells with peroxidase-linked antibodies and a precipitation reaction visualized with bright-field microscopy in place of fluorescence observations. In addition, Rieder and Alexander (1990) elegantly localized individual microtubules in glutaraldehyde-fixed cells with an antibody that fortuitously recognized glutaraldehyde-treated tubulin. This raises the intriguing possibility that primary antibodies raised against glutaraldehyde-treated antigens in conjunction with peroxidase-labeled secondary antibodies may prove to be a better means of localizing soluble proteins than the immunofluorescence techniques that are currently in use.

Finally, our observations emphasize the obvious importance of using other approaches to determine independently the properties and functional interactions of proteins with recognized cellular structures.

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**References**


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