

Mounting Technique Allows Observation of Immuno-Labeled Cells on Plastic Coverslips

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BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Mounting Technique Allows Observation of Immuno-Labeled Cells on Plastic Coverslips

BioTechniques 24:910-914 (June 1998)

Some cells grow very poorly on glass, if at all, unless its surface has been coated with a polymer (e.g., polylysine, collagen, agarose etc.) to permit cell adhesion (2). Unfortunately, these polymers can also nonspecifically bind primary or secondary antibodies, which greatly increases the background.

To address this problem, we have used Thermanox® plastic coverslips (Nalge Nunc International, Rochester, NY, USA) to grow 16HBE lung cancer cells. These coverslips are characterized by nonidentical faces, one of which is modified to increase cell adherence. This material is not recommended for immunofluorescence assays, as specified by the manufacturer, partly because of the strong autofluorescence of the plastic and partly because of its low light transmission capacity. After fixation and labeling with fluorochromes, the coverslips were carefully loaded onto a drop of an aqueous medium on the surface of a glass slide using a technique widely used for glass coverslips and referred to here as the standard protocol (Figure 1A). Alternatively, we have developed a technique in which the plastic coverslips are first fixed on the glass slide, loaded with a drop of aqueous medium and then covered with a glass coverslip (Figure 1B). This new technique considerably reduces the effects described above and allows the detection on plastic coverslips of specific antigens and DNA using any fluorochrome. Its main advantages are that it is simple, cheap and easy to carry out.

We have detected the Ki-67 antigen, a human nuclear protein whose expression is strictly associated with cell proliferation (3). This marker is widely used in routine pathology to measure the growth fraction of cells in tumors (1). The total number of cells was assessed by DNA staining, using either 4′,6-diamidino-2-phenylindole (DAPI) or chromomycin A3 (Sigma Chemical, St. Louis, MO, USA). 16HBE cells were grown on Thermanox coverslips in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 100 U/mL penicillin and 50 µg/mL streptomycin (Eurobio, Les Ulis, France) at 37°C in an atmosphere enriched with 5% CO_2. Cells at a density of 5–10 × 10^4 per coverslip (22 mm in diameter) were cultured for 72 h. After several washings in phosphate-buffered saline (PBS), pH 7.2, the cells were fixed for 4 min in 3% (wt/vol) paraformaldehyde, 1% (vol/vol) Triton® X-100 in PBS. The coverslips were then incubated for 30 min in PBS containing 3% (wt/vol) bovine serum albumin (BSA) (Sigma Chemical) and placed for 30 min with Mib1 monoclonal antibodies (Immunotech, Marseille, France) (6) diluted 1:20 in PBS containing 1% (wt/vol) BSA. The cells were washed in PBS (3 times, 5 min), and subsequently, biotinylated goat anti-mouse antibodies (Jackson, West Grove, PA, USA) (1:50 in PBS containing 1% [wt/vol] BSA) were applied for 30 min. After washing, the secondary antibody was detected

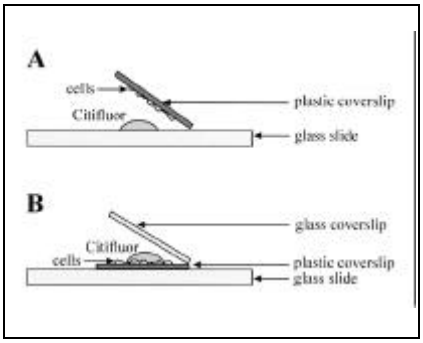


Figure 1. Schematic representation of the mounting techniques. After fixation and labeling with fluorochromes, the plastic coverslips were mounted in an aqueous medium. In the standard protocol (A), approximately 6 µL of Citifluor™ AF1 (Agar Scientific Ltd., Stansted, England, UK), a 90% glycerol solution containing an antifading agent, were applied to the glass slide. The plastic coverslip was then gently put in place to avoid trapping air bubbles between the aqueous medium and the cell surface. After removal of excess solvent, the mounting was sealed with transparent nail polish. In our new technique (B), the plastic coverslip was first fixed to the glass slide with nail polish, cells upwards, and a drop of Citifluor was gently put on their surface. A glass coverslip 22 mm in diameter was carefully adjusted onto the plastic coverslip and sealed as described for the standard protocol. Both mountings can be kept for several months at 4°C in the dark.

with streptavidin labeled with Texas Red (Amersham International plc, Little Chalfont, Bucks, England, UK) (1:25 in PBS containing 1% [wt/vol] BSA). The staining was performed for 15 min in the dark. Next, DNA was stained for 10 min either with 0.25 µg/mL DAPI in PBS or with 100 µM chromomycin A3 in PBS containing 150 mM $MgCl_2$. Plastic coverslips were then mounted as shown in Figure 1, either using a standard protocol (A) or according to our new technique (B) (see legend for details). Specimens were observed with an Axiophot® fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 40× Plan-Neofluar™ objective lens. Texas Red and DAPI stainings were visualized using appropriate filters. The slides were also analyzed using an MRC 600 Confocal Imaging System (Bio-Rad, Hercules, CA, USA) mounted on an Axioplan® microscope (Carl Zeiss) equipped with a 63×, 1.4 NA Planapochromat™ objective lens. As previously described (4), the 457-nm minor line of an argon laser was selected with a band-pass filter to excite chromomycin A3, while an HeNe laser producing a line at 543 nm was used to excite Texas Red. To obtain perfectly registered images, we have used a filter block composed of a dichroic mirror at 560 nm and a long-pass filter emission at 570 nm, so that observation of chromomycin A3 (DNA) and Texas Red (Ki-67 antigen) can be performed simultaneously.

When plastic coverslips are mounted according to Figure 1A, the cells are clearly observed by phase contrast microscopy (Figure 2A). However, a view of the same field analyzed by conventional fluorescence microscopy reveals a strong blurring effect that impedes the correct visualization of the Ki-67 antigen (Figure 2B). When protocol B is used (Figure 1B), both labels are clearly observed by phase-contrast microscopy (Figure 2, C and D) and by confocal microscopy (Figure 2, E–G). In this method, photons that both reach and are reemitted from the specimen cross glass, which has much higher transmission properties than plastic. Thus, it generates a high fluorescence signal, permitting a sharp and clear observation of both labels. The autofluorescence generated by the plastic coverslip is still present, but it occurs on a different plane, below the observed sample, and its contribution to the background is thus diminished. With protocol A, if autofluorescence were the only phenomenon impeding a correct visualization of the labels, this problem could be overcome by the use of confocal microscopy. However, blurred images were also obtained in this case (not shown). We believe that the nonidentical faces of the plastic coverslip might also provoke a light-scattering phenomenon that would greatly limit the contrast and the resolution of cell fluorescence when protocol A is used. With protocol B, this phenomenon is abolished, and it allowed us to study the proliferation rate of 16HBE cells. An average of 95% of Ki-67-positive cells (i.e., cycling cells) was found out of 10^3 cells observed. This value is in good agreement with data obtained for cancerous cells in culture (1). Figure 2D shows that the intensity and the localization of the Ki-67 antigens vary according to the phase of the cell cycle (7,8) and that even low staining levels, such as those observed during prophase, are detectable. With confocal microscopy, their detailed organization during interphase and metaphase (Figure 2, F and G) is revealed. The slight residual background observed in Panels C and D is totally absent in Panels E, F and G because of the pinhole effect of the confocal microscope. The optical sections thus collected have allowed us to perform highly detailed three-dimensional reconstructions whose quality is comparable to those previously obtained for cells grown on glass coverslips (5) (data not shown).

The mounting technique described here can thus be used very successfully, either with conventional fluorescence microscopy or with confocal microscopy. Furthermore, the wide range of fluorochromes that we have tested (DAPI, chromomycin A3, Texas Red and propidium iodide or fluorescein isothiocyanate [FITC] [data not shown]) confirms that this protocol can be extended to all available fluorochromes.

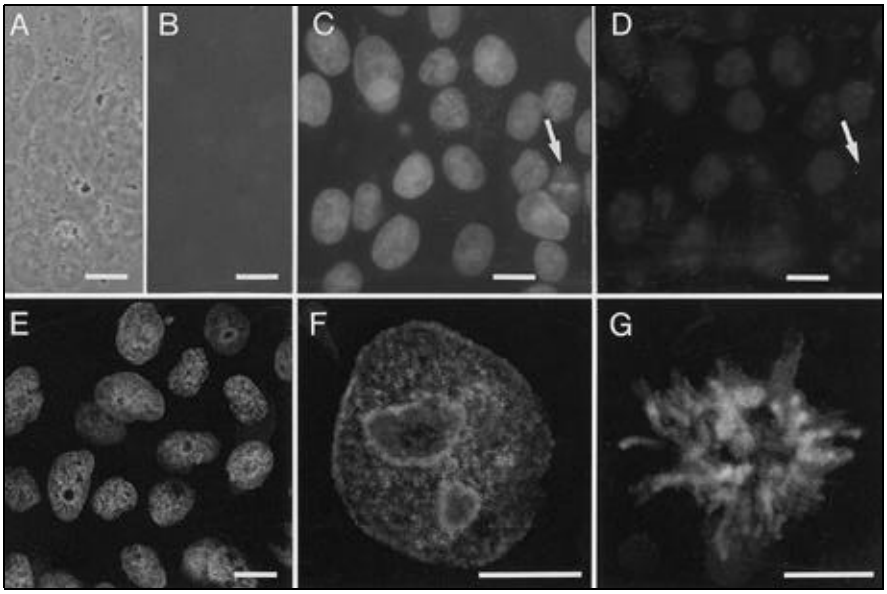


Figure 2. Fluorescence immuno-labeled cells mounted using either the standard protocol (A) or the improved protocol (B–G). 16HBE cells were mounted as in Figure 1A (A and B) or Figure 1B (C and D) and observed with an Axiophot fluorescence microscope. (A) Cells observed by phase-contrast microscopy; (B and D) Ki-67 antigen labeled with Texas Red; (C) DNA stained with DAPI. Arrows in Panels C and D show a Ki-67-negative cell. Analysis with a confocal laser-scanning microscope of cells mounted as in Figure 1B shows the double labeling of DNA (blue) with chromomycin A3 and of Ki-67 antigen (red) with Texas Red (E–G). (E) Cells were observed at the same magnification as those in Panels A–D. Views of individual cells during interphase and metaphase are displayed in Panels F and G, respectively. The bars represent either 10 µm (A–E) or 5 µm (F and G).

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Double Labeling for Whole-Mount In Situ Hybridization in Mouse

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Single-labeled whole-mount in situ hybridization is a valuable tool for studying gene expression during embryonic development. However, to understand possible interactions between two genes, it is advantageous if the expression patterns of both genes can be visualized in the same embryo. Several methods for sequential double labeling for whole-mount in situ hybridization have been described (6) and used successfully in *Drosophila* (1) and zebrafish (7). Similar methods have also been applied to large vertebrate embryos such as chicken (3,5) and *Xenopus* (8); however, in mouse it has been difficult to achieve good signal quality. Of the reagents and substrates available, alkaline phosphatase (AP) visualized with NBT/BCIP (Boehringer Mannheim, Indianapolis, IN, USA) is preferred because it provides a strong clean signal. For double labeling, detection of the second signal often relies on reagents other than AP or substrates with lower signal intensity than NBT/BCIP. Frequently, this results in high background and low signal intensity. However, using BCIP as the sole substrate for AP produces a turquoise signal (6) with an intensity matching the dark purple signal obtained with NBT/BCIP. We describe how these two substrates can be combined to provide an improved method for signal detection in double-labeled whole-mount in situ hybridization in mouse.

Pretreatment. The method used is modified from the methods in References 2 and 4. Embryos are dissected in diethyl pyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS) and fixed overnight in 100 mM MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 4% formaldehyde. After fixation, the embryos are washed twice in DEPC-treated PBS containing 0.1% Tween® 20 (PBST) (Sigma Chemical, St. Louis, MO, USA) and dehydrated in a series of 25%, 50% and 75% methanol in DEPC-treated PBST for 10 min each step. The embryos are then washed twice in ice-cold methanol and stored at -20°C. For rehydration, the embryos are taken through the same methanol concentration in reverse order. All steps are carried out on ice, and the embryos are allowed to sink in each concentration. After three washes in DEPC-treated PBST, the embryos are treated with proteinase K (10 µg/mL; Boehringer Mannheim) in DEPC-treated PBST at room temperature. The time of treatment depends on the size of the embryos and on the sites of gene expression. In cases in which the investigated genes are expressed in different tissues such as ectoderm and mesoderm, the time of treatment must be compromised; it must be long enough to reach the deeper mesoderm but short enough to avoid degradation of superficial tissues. In this study, we treated E12.5, E12.0 and E11.0 embryos for 50, 30 and 25 min, respectively (special care was taken when examining gene expression in the apical ectodermal ridge). Following proteinase K treatment, the embryos are fixed for 45 min in 4% paraformaldehyde (Sigma Chemical).

Hybridization. After fixation, the embryos are washed twice in hybridization solution (50% formamide [Sigma Chemical], 5× standard saline citrate [SSC], 2% blocking powder [Boehringer Mannheim], 0.1% Tween 20, 0.1% CHAPS [Sigma Chemical], 5 mM EDTA, 50 µg/mL heparin [Sigma Chemical] and 1 mg/mL yeast RNA [Sigma Chemical]). The steps are carried out at room temperature, and each time the embryos are allowed to sink in solution. The embryos are then incubated in hybridization solution at 65°C. After 1 h, the solution is changed, and the prehybridization is continued for another 3–4 h. RNA probes are labeled with DIG-11-UTP or fluorescein-12-UTP (both from Boehringer Mannheim), following the manufacturer's instructions. The differently labeled RNA products are added to the embryos (0.2–1 µg/mL digoxigenin-labeled RNA probe and 0.2–1 µg/mL fluorescein-labeled RNA probe), and hybridization is carried out overnight at 65°C. The post-hybridization washes are in decreasing concentrations of hybridization solution in 2× SSC (75%, 50% and 25%). Each step is carried out at room temperature for 10