Molecular Analysis of Animal and Plant Cells is Facilitated by Their Attachment to Collodion (Cellulose Nitrate) Films

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ABSTRACT

A procedure for the efficient transfer of cell monolayers, cultured on glass coverslips, to microscopy slides has been developed. This technique involves the coating of the upper surface of an ethanol-fixed cultured cell layer with a film of collodion dissolved in n*-amyl acetate. The dry collodion-coated cell layer can then be detached by rehydrating it for 1 h under water or phosphate-buffered saline and then carefully peeling it away from the coverslip using a pair of tweezers. Once a cell layer has been so mounted, it can be subjected to rough treatment such as proteolytic degradation (which greatly improves the signal-to-noise ratio in procedures like in situ hybridization [ISH] or primed in situ synthesis [PRINS]) without running the risk of cell detachment because of the partial or total degradation of the extracellular matrix. As an example of its application, we show a PRINS of telomeres from mouse fibroblasts. The high mechanical strength of collodion ensures that the structural integrity and morphology of the cell layer is maintained under experimental conditions where the collodion itself is insoluble. In addition to its use on cell layers, collodion can be used for the production of support films for* (i) *attaching suspension cell cultures,* (ii) *immobilizing cells normally cultured in suspension (such as tobacco BY2 cells or germinating tobacco pollen grains) or* (iii) *planting cryostat sections to microscopy slides. The value of this technique lies in its ease of use and the large number of different applications, in both the plant and animal fields of research, to which it may be applied.*

INTRODUCTION

Collodion is a form of cellulose nitrate (gun cotton) dissolved in *n*-amyl acetate. Its chemical structure is given in Figure 1 (24). The traditional solvent for the production of collodion solutions was 50:50 ethanol:diethyl ether (20); however, for safety reasons, *n*-amyl acetate is now used, as collodion solutions in ethanol/ether solvents are prone to explode upon drying. Upon evaporation of the solvent, a transparent film is left whose physical properties are determined by the nature of the solvents used. The higher the ethanol content in ethanol/ether mixtures, the softer and more flexible the film will be. High concentrations of ether result in very hard, brittle films (23). Gun cotton was discovered by Pelouze in 1838 by reacting cellulose with nitric acid, but it was not until 1846 that the technique for preparing collodion solutions was discovered by Schönbein (23,26).

Historically, collodion has been central to a number of key inventions, ranging from the discovery of Rayon (artificial silk) by a technician of Louis Pasteur in the 1870s (2), to its use in the production of nitrocellulose filters (24) and dialysis membranes (10,18). However, the most relevant applications to our current paper were as follows. In 1851, Frederick Scott Archer invented the collodion "wet-plate" photographic process, whereby silver halides were suspended in a collodion solution (23). These photographic emulsions were then painted onto glass plates and exposed; however, collodion does not stick to glass, and the collodion negatives would often detach from the glass. It was not until the invention of the gelatine "dry plate" method in 1871 by Maddox that this problem was solved. Maddox found that coating the glass with gelatin/chrome alum provided a surface to which the collodion would bind very strongly, and the gelatin in turn was tightly bound to the glass (23). Between 1850 and 1930, collodion was used as a surgical dressing for sealing cuts and minor wounds because it would stick easily to human tissues and dry to form a soft, flexible membrane that was able to hold the edges of the wound together without breaking or permitting bacteria to enter (25). Collodion is also used for the embedding of large or brittle tissues for sectioning, where paraffin is not appropriate (19). The major use for collodion solutions today is in the production of films for

use in transmission electron microscopy (EM) (4,16,17,21).

Collodion dissolved in *n*-amyl acetate is ideal for use in EM because it produces an extremely strong film of constant thickness and does not involve the highly explosive solutions previously produced when ethanol/ether solvents were used. We have made use of collodion's high porosity and affinity for biological material to develop an extremely simple new procedure for the mounting of tissue sections and for the transfer of cell monolayers from coverslips to slides. Given the use of collodion in EM we suspect that this procedure may also be of value to researchers wishing to view cell monolayers under the electron microscope.

There are many extracellular matrices (ECM) that are suitable for the culture of mammalian cells. These matrices are generally protein-based supports such as collagen, gelatine (19) or bovine serum albumin (BSA) (6) or carbohydrate matrices such as soft agarose for the culture of tumor cells (22). In addition, peptide polymers like poly-L-lysine are useful for the mounting of tissue sections on slides. Such supports hold mammalian cells extremely efficiently but are less effective with plant cells. This work began with our search for a way of "glueing" plant cryostat sections onto microscopy slides with an optically transparent "glue". It is known that tissue blocks embedded in collodion may be sectioned by sticking the collodion block onto a wooden holder using collodion dissolved in ethanol/ether (19). Given that this is strong enough to hold the sample during sectioning, we reasoned that the collodion glue might provide an effective way of attaching plant material to slides. The only problem was that the collodion itself does not stick to glass, so the sections tended to detach with the collodion film. This problem is the same as that of the "wet-plate" collodion method discussed above. The use of the gelatine "dry-plate" solution also solved our problem, and the combination of gelatine-coated slides and collodion-mounted sections provided a simple yet effective means of attaching plant material to slides. The same process also works well for attaching *para*-formaldehyde-fixed plant suspension cell cultures, such as tobacco BY2 cells, to slides without the risk of their moving or being washed away.

More recently, we have been performing immunohistochemistry and primed in situ synthesis (PRINS) on mouse C3H10T½ cells (13). We found it extremely difficult to find dividing cells on our slides after antibody staining because they often became detached during the staining and washing steps. We also found it difficult to perform PRINS labeling on dividing cells because they always showed an extremely high cytoplasmic fluorescence. We reasoned that the application of a layer of collodion to the upper surface of the cell layer would prevent the detachment of dividing cells during immunohistochemistry and would enable us to use proteinase K to digest cellular proteins, thus improving the signal-to-noise ratio in the PRINS without the risk of detaching the cells because of degradation of the ECM.

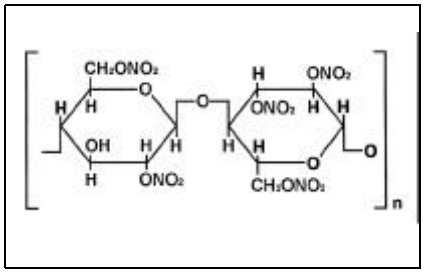

Figure 1. Chemical structure of collodion. Collodion is composed of fibers of cellulose nitrate (24). The basic building block is a disaccharide that has been polymerized into long chains. One sugar of this disaccharide has every "-OH" group replaced by "-ONO_2", whereas the other sugar still retains an "-OH" group on carbon 4.

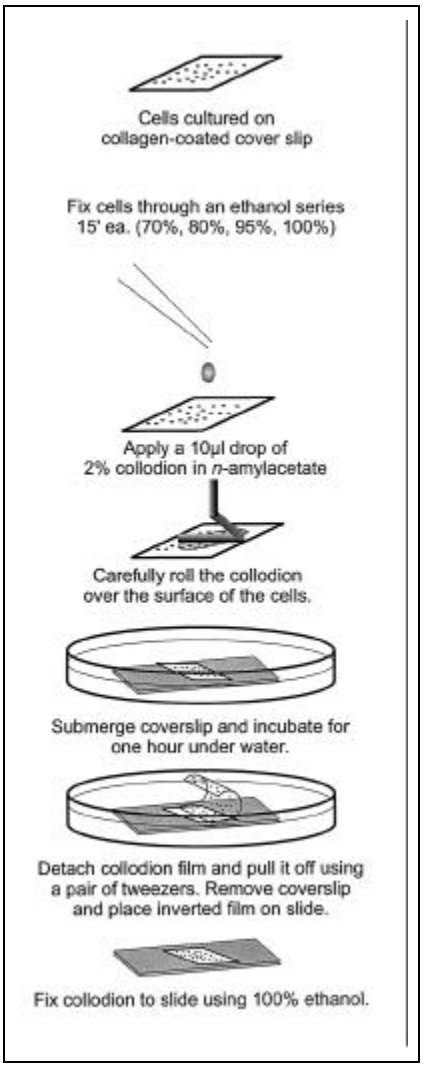

Figure 2. Collodion-coating and cell transfer protocol. The steps required for the application of collodion and the removal of the cell layer are shown in a pictographic form to facilitate the application of this procedure.

Table 1. Solubility of Collodion in Common Solvents

Solvent Tested	Collodion Solubility	Solvent Effect
Water	Insoluble	The collodion appears unaffected by immersion for 1 week in water.
PBS	Insoluble	The collodion appears unaffected by immersion for 1 week in water.
4% *para*-formaldehyde/PBS	Insoluble	The collodion appears unaffected by immersion for 1 week in water.
Diethyl ether	Insoluble	The collodion becomes very hard and cloudy in appearance but does not dissolve after 5 h.
Chloroform	Insoluble	The collodion becomes very hard but does not discolor or dissolve.
Isopropanol	Insoluble	The collodion appears unaffected by immersion for 5 h in isopropanol.
70% ethanol	Insoluble	The collodion appears unaffected by immersion for 5 h in isopropanol.
100% ethanol	Slightly soluble	The collodion softens over 2–3 min but does not lose its shape or dissolve by immersion for 5 h.
Methanol	Soluble	The collodion becomes very soft and completely dissolves in 10 min.
Acetone	Soluble	The collodion becomes very soft and completely dissolves in 10 min.
50:50 Ethanol/diethyl ether*	Soluble	The collodion becomes very soft and completely dissolves in 10 min.
n-amyl acetate	Very soluble	The collodion becomes very soft and completely dissolves within 2–3 min.

The solubility of collodion has been tested in the solvents listed. This was done by placing a 1-mL micro-collodion bag (Sartorius, Goettingen, Germany) in 15 mL of the desired solvent and incubating the mixture in a 50-mL polypropylene tube for 5 h at room temperature. Solvent mixtures can have properties that are different from those of either of the solvents in isolation. For example, collodion is insoluble in both diethyl ether and ethanol but it is very soluble in ethanol/ether mixtures.

The mechanical properties of the collodion film deposited after the evaporation of the ethanol/ether depend upon the ratio of these solvents used. As the proportion of ether is reduced, the deposited film becomes softer and more flexible; increasing the ether content gives very hard, brittle, hydrophobic films.

***CAUTION:** Extreme care should be taken when evaporating ethanol/ether/collodion solutions, as this is a highly explosive mixture (11). If such solutions must be used, then they should be evaporated in a humid atmosphere (>80% relative humidity) to reduce the risk of explosion.

The experimental techniques for PRINS or in situ hybridization (ISH) are often much more sensitive and give lower background signals if the cells are first subjected to protease treatment (5,7,14). However, given that the cells are attached both to each other and to the coverslip by proteins, this treatment must be extremely mild; otherwise, the cells will detach and be lost (5). Collodion provides a much better support for such procedures since it is composed of cellulose nitrate (Reference 24; Figure 1) and as such is not susceptible to pro-

teolytic degradation. Thus, fixed material can be digested without the risk of the cells detaching.

MATERIALS AND METHODS

Collagen Coating of Coverslips

Glass coverslips (18- × 18-mm; Menzel-Gläser, Sondheim, Germany) were cleaned extensively using 70% ethanol and allowed to air-dry. One hundred fifty microliters of collagen solution (1 μg/μL) were pipetted onto the center of the coverslip and spread out, using a 20-mm-wide glass bacteria-spreader (see Collodion Treatment) and allowed to air-dry.

Gelatine Coating of Slides (Subbing)

Glass microscopy slides (76- × 26-mm; Menzel-Gläser) were "subbed" with a gelatine/chrome alum solution, which was formed by dissolving 0.5 g gelatine in 100/mL boiling water and then adding 0.05 g potassium chromium (III) sulfate to the solution. The slides were then dipped into the hot (ca. 65°C) solution and allowed to air-dry.

Cell Fixation

All of the standard procedures for cell-fixation work well with this procedure. We have tried ethanol, methanol and *para*-formaldehyde and found no noticeable differences in the behavior of the collodion. However, in this paper, we will show cells that have only been ethanol-fixed, as we have found that the PRINS reaction works best on samples that have been fixed with chemicals that do not cause too much chemical cross-linking.

Cell Culture

Mouse C3H10T½ cells, obtained from ATCC (Rockville, MD, USA) were cultured to confluency at 37°C for 24 h in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum, on collagen-coated coverslips.

Tobacco (*Nicotiana tabacum* L. *cv.* bright yellow 2) BY2 cells (1) were cultured to an A_{600} of 0.8 in Linsmaier and Skoog liquid medium (12), supplemented with 0.2 μg/mL of the auxin analogue 2,4-D (Sigma Chemical, St. Louis, MO, USA), for 48 h at 28°C. One hundred microliters of BY2 cells were then ethanol-fixed by passing them through a cold (-20°C) ethanol series consisting of 80%, 95% and 100% ethanol (for 15 min each) and then either attached to a coverslip using collodion or resuspended in phosphate-buffered saline (PBS) and viewed directly.

Collodion Treatment

The cells were fixed by the removal of the growth medium followed by incubation in cold (-20°C) 70% ethanol for 15 min. The cells were then dehy-

drated by passing them through a cold (-20°C) ethanol series consisting of 80%, 95% and 100% ethanol (15 min each). The cells were then allowed to air-dry, and 15 µL of 2% collodion in *n*-amyl acetate (Electron Microscopy Sciences, Ft. Washington, PA, USA) were spread over the dry cell layer using a 20-mm-wide glass spreader (Figure 2), self-made from a 2-mm-diameter glass rod, duplicating the coat-hanger shape of a regular bacteria-spreader. The collodion was allowed to air-dry, and the coverslip was submerged, cell layer uppermost, in a 9-cm petri dish containing 20 mL of either water or PBS and a submerged, gelatin/alum subbed, 76- × 26-mm microscopy slide (Menzel-Gläser). After approximately 1 h, the collodion had hydrated, taking on a slightly shiny appearance, with small surface ripples being visible. At this stage, one edge of the film was grasped between the ends of a pair of blunt-nosed tweezers and gently pulled away from the coverslip (Figure 2). The entire film was detached as a single sheet, becoming inverted in the process so that the cell layer was uppermost. The collodion film was positioned over the submerged microscopy slide, and the excess water was removed using a 25-mL pipet (Falcon®; Becton Dickinson Labware, Bedford, MA, USA) so that the collodion film landed on the surface of the slide. The collodion was attached onto the slide by applying either 1 mL of ice-cold 100% ethanol (in the case of water-hydrated samples) or a cold ethanol series of 70% ethanol for 15 min followed by 100% ethanol (in the case of PBS-hydrated samples). The ethanol makes the collodion "sticky" so that it will bind to the slide after it has been allowed to air-dry. After the ethanol treatment, the collodion is fairly resistant to rehydration and will not detach from the slide. **CAUTION:** Note that collodion is soluble in certain organic solvents (see Table 1), so care should be taken when exposing samples to nonaqueous solvents. If samples need to be treated with solvents other than those listed in Table 1, then these should be tested on control collodion films before risking valuable cells.

Staining of Cells

To determine the effect of collodion treatment on cellular morphology, one

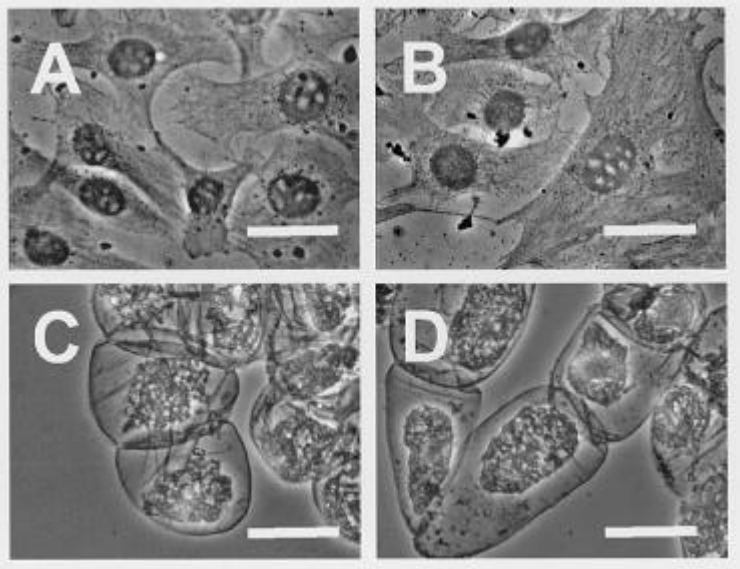

Figure 3. The effect of collodion on cell morphology. (A) An ethanol-fixed mouse fibroblast cell layer before the collodion treatment. The cells are attached to a collagen matrix on an 18- × 18-mm coverslip. (B) A similar view of mouse fibroblast cells after they have been coated with collodion and transferred to a clean microscopy slide. (C) Tobacco BY2 cells in PBS (without attachment to the slide). (D) Tobacco BY2 cells mounted on a collodion matrix. The scale bars are 10 µm.

batch of fixed cells was transferred to a collodion membrane, while a second was left attached to the collagen support. The cells were then stained using the Giemsa-May-Grünwald procedure (3). Giemsa solution was obtained from Fluka (Buchs, Switzerland), and May-Grünwald solution was obtained from Merck (West Point, PA, USA). This stained the nuclei blue and the cytoplasm red-orange and facilitated the visualization of cells under phase-contrast light microscopy.

Although the collodion membrane was also weakly stained by this procedure, it was still possible to obtain a good contrast between the cells and the background.

Incubating the cells with 0.1 µg/mL 4′,6-diamidine-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) for 30 min followed by a wash in PBS, stained the nuclei of the cells.

Primed In Situ Synthesis

Our polymerase chain reaction (PCR) buffer (8) for the PRINS made use of the specificity enhancement properties of tetramethyl ammonium chloride. This chemical works, in conjunction with ammonium ions, to increase the melting temperature of perfect-match AT base pairs to approximately that of GC base pairs without stabilizing mismatch base pairs (9). This has two beneficial effects: *(i)* it makes the PCR more stringent, and *(ii)* it makes the annealing temperature of the primer length, rather than sequence, dependent (9). This allowed us to design optimum conditions that would work well for primers of a given length, irrespective of their sequence composition.

Our protocol for PRINS is somewhat different from the classical procedure in that we do not possess a thermal cycler built to take slides. Instead, we have adapted an early thermal cycler for this purpose by covering the heating block with aluminium, placing the slides on this and then covering it all with aluminium foil to ensure even heating of the slides. A detailed protocol is given as a guide for people who wish to try PRINS on nonspecialist thermal cyclers. The important point is simply to demonstrate that dividing cells are not detached from collodion films by proteinase K treatment and that this removes the nonspecific staining in these cells.

PRINS was performed in two different ways. In the first experiment, collodion-immobilized cells were mounted on a glass slide and 20 µL of PRINS solution [67 mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 170 µg BSA, 16.6 mM $(NH_4)_2SO_4$, 500 µM tetramethyl ammonium chloride (Sigma Chemical); 50 µM each of dGTP, dCTP, dATP (Amersham Pharmacia Biotech, Uppsala, Sweden) and Texas Red® isothiocyanate-dUTP (Boehringer Mannheim, Mannheim, Germany); 1 µM telomer-specific PRINS primer (5′-TTAGGG-TTAGGGTTAGGGTTAGGG-3′); 1% Triton® X-100 (to permeabilize the cells); and 10 U of *Taq* DNA polymerase] were applied to the sample. The sample was then sealed under an 18- × 18-mm coverslip using clear nail varnish (Procter & Gamble, Geneva, Switzerland) and transferred to a pre-heated aluminium plate on the heating block of a Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) and covered with aluminium foil to ensure good thermal conduct through the

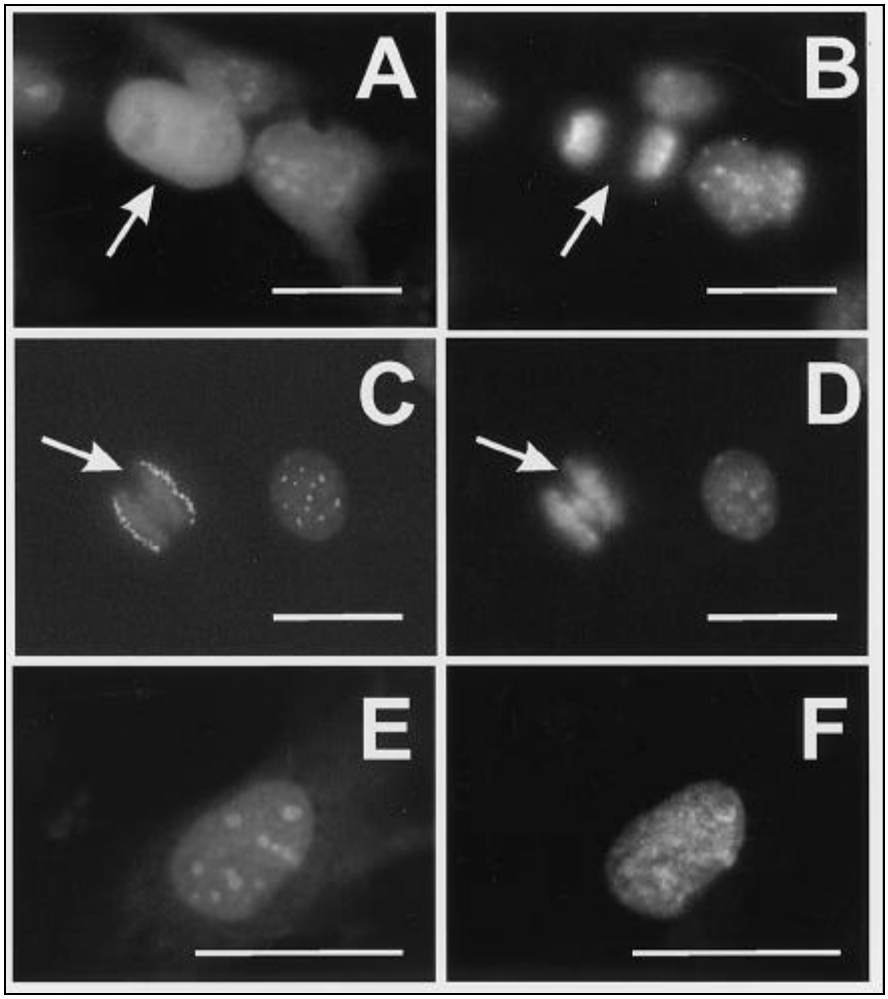

Figure 4. The effect of proteinase K treatment on the PRINS-labeling of telomeric sequences. (A) A view from a PRINS experiment using telomere-specific primers to incorporate Texas Red isothiocyanate-dUTP into nuclear DNA from collodion-mounted C3H10T½ cells. The arrow indicates a dividing cell. Note the intense cytoplasmic background masks the PRINS signal effectively. The number of telomeric spots present in non-dividing cells was somewhat higher than the apparent number, because those that lie out of the plane of focus cannot be seen in these pictures. (B) DAPI staining of the same view with the chromosomes clearly visible. (C) A second dividing cell, however, it was treated with proteinase K before the PRINS experiment. The arrow indicates the dividing cell. The proteinase treatment removed most of the nonspecific cytoplasmic staining. (D) DAPI staining of Panel C. The concentration of proteinase K used in these experiments was 0.1 µg/µL. The scale bars are 10 µm.

sample. Primer annealing and extension were performed in a single cycle as follows: 94°C for 10 min; ramp cooling from 94°–55°C over 10 min; 55°C for 20 min and then 72°C for 40 min. Submerging the slide in ice-cold PBS containing 10 mM EDTA and 1% Triton X-100 and removing the nail varnish and coverslip with a toothpick, stopped the reaction. The PBS/EDTA/Triton solution was then replaced with a fresh solution and incubated on ice for 20 min followed by a second wash step for a further 20 min. The samples were then covered with 30 µL VECTASHIELD™ Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and a coverslip and then viewed under a Model DM-1RBE Fluorescence Microscope (Leica, Unterentfelden, Switzerland).

For the second experiment, the sample was first overlaid with 100 µL proteinase K, dissolved in water (0.1 µg/µL) and incubated at room temperature for 10 min (5). The proteinase K was removed by washing the slide twice with ice-cold PBS for 20 min. The PRINS solution was then applied to the sample, and it was treated as outlined above.

RESULTS

Effect of Collodion Transfer on Cell Morphology

Cell layers were visualized post-fixation by phase-contrast light microscopy after Giemsa-May-Grünwald staining (this staining was performed to improve the contrast of our photographs showing cell morphology. It was not used for the PRINS experiments). Figure 3A shows ethanol-fixed cells grown on collagen. Figure 3B shows a similar view of ethanol-fixed cells that have been transferred onto a collodion support. It is clear from Figure 3 that the collodion treatment has no significant effect on either cellular or nuclear morphology when compared with those cells that had been fixed but not transferred. A suspension of tobacco BY2 cells was also applied to an 18- × 18-mm coverslip, and the growth medium was carefully blotted away. The cells were then dried through an ethanol series (as above) and coated in collodion. The procedure was as described for animal cells. Figure 3C shows BY2 cells mounted on glass in PBS. Figure 3D shows a similar view of BY2 cells, but in this case, they had been attached to a collodion membrane. As can be seen, the collodion does not have a significant effect on plant cell morphology either.

PRINS-Labeling of Chromosome Telomeres in Mouse C3H10T½ Cells

Figure 4A shows a fluorescent micrograph of a dividing cell without pretreatment with proteinase K. Figure 4B shows DAPI staining of the same view. Note that in Figure 4A, the intense cytoplasmic fluorescence of the dividing cell totally masks the labeled ends of the chromosomes. We believe that this nonspecific signal is caused by the binding of labeled nucleotides to the cell as proteinase digestion, before PRINS-labeling, abolishes this nonspecific fluorescence and permits the chromosomes to be seen more clearly (Figure 4C). The reduced fluorescence in the PRINS-labeling, after proteinase treatment, permits the clearer interpretation of such experiments. Figure 4D shows DAPI staining of Figure 4C. Figure 4E shows PRINS-labeling of cells that were not treated with either collodion or proteinase K. No dividing cells were observed. Figure 4F shows DAPI staining of Figure 4E.

DISCUSSION

We present here a simple procedure for the transfer of intact cell layers from coverslips to slides. Such a treatment facilitates downstream applications such as PRINS by binding cells irreversibly to a cellulose nitrate film even after prolonged exposure to high concentrations of proteinase K. This technique may also be useful in any other experimental protocol where the tight, effectively irreversible, binding of cells to a non-proteinaceous membrane is desired. We have made use of this in immunohistochemical experiments with plant cells. This technique was used for immobilizing fixed germinating tobacco pollen so that antibody washes could be performed without breaking the fragile pollen tube (15). It should be stressed that we have maintained both plant and animal collodion-mounted cells layers in beakers of PBS for several weeks without any apparent loss of cells (the longest time we have tested is four weeks). In fact, the only way to separate the cells from the collodion membrane was to redissolve the collodion in one of the suitable solvents listed in Table 1. The best way to attach the collodion film to a slide is by the gelatin "dry plate" procedure, where the collodion film is placed on a gelatin/chrome alum-subbed slide and dried by washing it in 100% ethanol. The collodion support matrix underneath the cells permits the use of enzymes, such as proteases, without the

risk of cell detachment. The matrix has also been used for improved mounting than can be obtained by classical procedures of the following: *(i)* plant suspension cells (tobacco BY2 cells; see Figure 3, C and D), *(ii)* fixed germinating tobacco pollen (15), *(iii)* tobacco cryostat sections (data not shown) and *(iv)* cultured nerve cells (S. Kaech, personal communication). The simplicity and reproducibility of this procedure make it amenable to a wide variety of experimental procedures. We feel sure that it may be profitably used to facilitate many experimental protocols involving cultured cells or histological sections in both the plant and animal fields.

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