Charged nylon membrane substrate for convenient and versatile high resolution microscopic analysis of *Escherichia coli* & mammalian cells in suspension culture

Manoj Raje · Rohan Dhiman · Sekhar Majumdar · Taposh Dass ·
Kanak L. Dikshit · Ramandeep Kaur

Received: 16 June 2006 / Accepted: 4 October 2006 / Published online: 3 November 2006
© Springer Science+Business Media B.V. 2006

Abstract Preparation of isolated cells and microorganisms for ultrastructural examination always provides a challenge in terms of adequate immobilization of the cells and prevention of subsequent sample loss and damage during various steps of sample processing. Using a positively charged nylon membrane substrate we demonstrate that it is possible to easily immobilize and retain a sample of isolated cells in culture for a wide variety of microscopy-based techniques. Radiolabelled *E. coli* cells when immobilized on the charged membrane were seen to be highly resistant to detachment when subjected to the normal sample processing procedures associated with microscopy. In contrast cells on regular millipore membranes were rapidly lost during sample preparation. We demonstrate the utility of charged nylon membranes for a wide variety of microscopy based analysis including scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), TEM

based immunogold labelling, laser confocal microscopy and SEM based elemental analysis.

Keywords Charged membrane · Electron microscopy · Processing · Immobilization · Isolated cells

Introduction

The preparation of cells in suspension for a wide variety of microscopy-based analysis by a single procedure raises considerable problems. Small samples consisting of freely floating cells usually requires several preparative steps. The literature in this area usually suggests the use of either centrifugation of samples (van de Sandt et al. 1985) or their entrapment in membranes (Fletcher and Floodgate 1976; Kai et al. 1999) or coagulable fluids (Hirsch and Fedorko 1968; Kaps and Burkhardt 1985). A method utilizing porous cellulose capillary tubes to immobilize suspensions of nematodes, paramecia and bacteria (Hohenberg et al. 1994) as well as plant cells (Tiedemann et al. 1998) for high-pressure freezing and subsequent processing for transmission electron microscopy by freeze-substitution has also been described in literature. Repeated and high-speed centrifugation or washing and resuspending procedures may cause mechanical damage and significant loss of sample into the

M. Raje (✉) · R. Dhiman · S. Majumdar ·
K. L. Dikshit · R. Kaur
Institute of Microbial Technology, Sector 39 A,
Chandigarh 160036, India
e-mail: manoj@imtech.res.in

T. Dass
All India Institute of Medical Sciences, New Delhi
110029, India

washing medium. In most cases the procedure renders the sample suitable to study only by a limited range of analytical microscopy techniques.

Here utilizing examples of prokaryotic as well as eukaryotic cells in suspension we demonstrate the utility of immobilization on charged nylon for studying the samples using a broad spectrum of modern microscopic tools.

Materials and methods

Nylon membrane and cell samples

Positively charged nylon membrane filters (SNNPZ, 0.45 μm pore size) were purchased from Advanced Microdevices Ambala, India.

Escherichia coli DH5 α cells grown to mid log phase in LB medium and THP-1 (a human monocytic cancer cell line obtained from ATCC) cell line maintained in RPMI-1640 medium with 10% foetal bovine serum were used as a representative samples of cells in suspension. All chemicals were of analytical grade.

Evaluation of SNNPZ membrane capability to retain cells through sample-processing steps.

Small pieces of SNNPZ membrane (1 cm^2) were cut and immersed in PBS for 1 h. *E. coli* cells were metabolically labelled with S^{35} methionine obtained from BARC (BARC, India) washed three times with ice cold Dulbecco's phosphate buffered saline (PBS) and finally resuspended in 100 μl of the same buffer ($\text{OD}^{600\text{ nm}} = 10$). Five microlitre aliquots of cells were spotted on to small pieces of SNNPZ membrane, which were then rinsed three times with cold PBS and processed as tissue samples for electron microscopy. At different steps during the processing, triplicate sets of filter papers were removed and counted in a β -counter. An evaluation of the charged membranes ability to retain cells was computed from the percentage of radioactivity retained on the membrane as compared to prior to processing of the filters. For comparison a set of *E. coli* cells were immobilized on pieces of Millipore filter (Millipore Co., Bedford, MA) and processed in parallel sets of experiments.

Processing of cells on charged membranes for routine TEM and SEM

Escherichia coli cells immobilized on SNNPZ membrane were washed thrice with ice cold PBS, fixed for 2 h in modified Karnovskys fixative, post-fixed in 2% OsO_4 and dehydrated in either graded acetone or ethanol as per the following protocol:

30% Acetone/alcohol 30 min at 4°C

50% Acetone/alcohol 30 min at 4°C

70% Acetone/alcohol 30 min at 4°C

90% Acetone/alcohol 30 min at room temperature

95% Acetone/alcohol 30 min at room temperature

100% Acetone/alcohol 30 min at room temperature (three changes)

Acetone dehydrated samples were infiltrated with epoxy resin (EMS) and embedded in Beem capsules. Polymerization was carried out at 60°C for 72 h. Ultrathin sections were cut and picked up on copper grids, contrasted using uranyl acetate as well as lead citrate and observed in a JEOL 1200 EXII, transmission electron microscope.

Alcohol/acetone dehydrated samples were also processed for scanning electron microscopy by the freeze drying procedure utilizing tertiary butyl alcohol as an intermediate fluid (Inoue and Osatake 1988). Membranes were subsequently mounted on aluminium stubs using silver paint and sputter coated with gold. Cells were observed and photographed using a S-260, Leica Cambridge scanning electron microscope.

Processing of cells on charged membranes for immunogold labelling TEM

Escherichia coli DH5 α cells harbouring the plasmid PUC8: 15 expressing *Vitreoscilla* haemoglobin protein (VHb) (Dikshit and Webster 1988) were used as a source of antigen. Cells from stationary phase were harvested, washed with PBS and resuspended in the same buffer as above. Five microlitres of cell suspension were spotted onto a piece of SNNPZ membrane, which was then processed for electron microscopy.

Samples were fixed for 30 min in 1% glutaraldehyde (EM Sciences; Ft Washington, PA) + 4% paraformaldehyde (Polysciences; Warrington, PA) constituted in PBS, dehydrated in alcohol and processed for embedding in LR white resin (EMS) at 60°C for 24 h. Ultrathin sections were cut, flattened with xylene vapour, collected on nickel grids, processed for immunogold labelling using polyclonal antiserum against VHb protein raised in rabbit. Antibody dilutions and immunogold labelling was carried out as described previously (Ramandeep et al. 2001). Immunogold labelled grids were contrasted with 2% aqueous uranyl acetate and observed in a JEOL 1200 EXII transmission electron microscope.

Sample preparation for LASER confocal microscopy

Small pieces of charged membrane were sterilized in a steam autoclave and placed into 6 well tissue culture plates (Nunc). Membranes were soaked for 2 h in 2 ml of medium. THP-1 cells were seeded into the medium and allowed to attach overnight at 37°C in a 5% CO₂ atmosphere. The next day the membranes were briefly washed with PBS and fixed in 2% paraformaldehyde (constituted in PBS) for 15 min. After three rinses with PBS the complete membranes were double stained with DAPI as well as TRITC-Phalloidin (Molecular Probes) as per the manufacturers instructions. The membranes, with cells adherent on them were then mounted on glass slides using Fluomount-G (Southern Biotechnology Associates) and examined in an LSM-510 META LASER confocal microscope (Carl Zeiss).

Sample preparation for observation of cells by AFM

Cells immobilized on SNNPZ membranes and freeze dried for SEM study as described above were directly mounted on sapphire holders without sputter coating. Cells were observed in a Solver PRO AFM (NT-MDT) atomic force microscope in semicontact mode.

EDAX analysis of nylon membrane

EDAX spectrum of representative samples of SNNPZ membrane was collected using an INCA200EDAX attached with a Leo S430 scanning electron microscope.

Results

Ability of charged nylon membrane to adhere with cells was tested by allowing radiolabelled cells to adhere to the surface of the filters and then subjecting the membrane to the various sample processing steps microscopy such as repeated washing, exposure to: fixatives, organic solvents, dehydration and drying. Results are presented in Table 1. About two thirds of total cells loaded were found to be retained after osmium post fixation. Even after subsequent dehydration upto 100% with acetone or alcohol over half of the cells remained adherent to the charged membrane substrates while only around 6% were retained onto Millipore membranes. After freeze drying for SEM studies, SNNZ membrane samples retained around twenty

Table 1 Retention of cells on charged nylon membrane after various sample processing steps

Sample processing step	Radioactivity retained as percentage of initial (mean ± SD)*			
After washing and aldehyde fixation Post fixation with OSO ₄	Cells on SNNPZ		Cells on Millipore membrane	
	69.13 ± 4.55		34.29 ± 6.45	
70% Dehydration	65.83 ± 7.40		31.22 ± 9.91	
	Processing with acetone	Processing with alcohol	Processing with acetone	Processing with alcohol
100% Dehydration	52.73 ± 8.75	55.42 ± 9.71	14.11 ± 3.90	19.22 ± 5.31
Freeze drying through tertiary butyl alcohol	51.39 ± 3.07	53.31 ± 7.48	6.02 ± 5.95	6.65 ± 4.91
	18.15 ± 2.56	23.04 ± 4.92	<5	<5

* *n* = 5

percent of initially loaded radioactivity. In comparison cells immobilized on Millipore membranes were seen to rapidly come off after the freeze drying step with only about 5% of initial radioactivity remaining. The loss in radioactivity from membranes could also be due to leakage of radiolabelled molecules from damaged adherent cell fragments or a consequence of extraction during the dehydration process. We did not observe many disrupted cells in the remaining adherent population, which seemed to comprise primarily of cells with intact membranes without any discernible distortion. Previous studies by other groups, utilizing adherent cell monolayers on discs of membrane filters, combined with freezing and freeze-substitution fixation, demonstrated both membranous and filamentous elements of the cytoplasm are evenly preserved and well contrasted (Morphew and McIntosh 2003).

The observation that a significant number of cells immobilized on SNNPZ membranes had been retained was confirmed when the nylon membranes were examined by SEM and TEM (Fig. 1a, b). Ultrathin sections of the immobilized cells embedded in epoxy resin showed that a layer

of cells adheres tightly to the charged membrane surface. Immunogold labelling of *E. coli* cells demonstrated a high level of labelling on the cells with low levels of background (Fig. 2). Immobilized cells were also clearly discernible using an atomic force microscope (Fig. 3). To check if the SNNPZ membrane could be used for fluorescence microscopy, DAPI and TRITC labelled THP-1 cells were examined. The signal from the nucleus (DAPI) and cytoplasm (TRITC 1-phalloidin labelled microfilaments) is clearly visible with almost no background fluorescence in both channels (Fig. 4a–c). In addition no disruption of the cytoskeletal (actin) filaments was observed. EDAX analysis of the membrane samples showed practically no presence of any high atomic number elements. Only a chloride peak is seen (Fig. 5) which is most probably due to residue leftover from the bleaching process during manufacture.

Discussion

Sample preparation of cells in suspension for microscopic examination has always provided a

Fig. 1 SEM (a) and TEM (b) of *E. coli* cells retained after all sample processing steps. Individual cells, tightly adherent individual cells are clearly discernible on the charged nylon membrane

Fig. 2 Immunogold labelling of cells on SNNZ filter membrane. The gold label on cells is clearly seen. Minimal background labelling is observed

Fig. 3 Semi-contact mode atomic force microscopy (AFM) image of bacterial cells immobilized on charged filter paper. The scale bar is given along the x and left hand y -axis. On the right hand y -axis is present a greyscale representing the z -axis depth of the image

Fig. 4 THP-1 cells double stained with TRITC-Phalloidin (**a**) and DAPI (**b**). The Phalloidin stained microfilaments form a band at the cell periphery circumscribing the DAPI stained nucleus as seen in the merged image (**c**)

challenge. To obtain representative information from microscopic examination of biological specimens it is necessary to ensure that there is a minimal loss as well as lack of any distortion occurring in the sample material. Existing methods to achieve this goal have usually depended upon centrifugation and pre-embedding of the sample in a matrix.

As the processing of samples for certain microscopic techniques can be quite tedious it is important that sample loss be kept to a minimum so that sufficient material is retained for getting a proper representation of the original sample as well as collecting sufficient data for statistical evaluation. In addition it may also be necessary to subject the same sample to

Fig. 5 Representative sample of nylon membrane as observed in SEM (a) along with the EDAX spectrum obtained (b). As can be seen apart from the peaks for low

atomic number elements of carbon and oxygen only a chloride peak is seen, possibly from the residue left in bleaching of the filter during manufacture

examination by a wide array of microscopic evaluation tools.

Practically all biological cells demonstrate the presence of a net negative charge on their surface and this property has been used to immobilize cells onto positively charged poly- Lysine coated glass coverslips for subsequent microscopic observation in the past (Mazia et al. 1975). Samples immobilized on glass are suitable only for a limited range of microscopy based evaluation methods. Though the method involving immobilization in porous cellulose capillary tubes provides excellent results for TEM analysis of microorganism ultrastructure it requires a high pressure freezing apparatus that is relatively expensive and its utility for other modes of microscopic analysis like confocal and atomic force microscopy is not clearly documented. In this study we have demonstrated the utility of a charged nylon membrane for rapid immobilization of suspension samples, which are then easily

amenable to a wide range of microscopy based analytical techniques without major losses of material.

Acknowledgements The skilful technical assistance of Mr. Anil Theophilus is gratefully acknowledged. R. Dhiman was the recipient of CSIR research fellowship. This manuscript is IMTECH communication number No. 05/2006.

References

- Dikshit KL, Webster DA (1988) Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene 70:377–386
- Fletcher M, Floodgate GD (1976) The adhesion of bacteria to solid surfaces. In: Fuller R, Lovelock DW (eds) Microbial ultrastructure the use of the electron microscope. Academic Press, London, pp 101–107
- Hirsch JG, Fedorko ME (1968) Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and postfixation in uranyl acetate. J Cell Biol 38:615–627

- Hohenberg H, Mannweiler K, Muller M (1994) High-pressure freezing of cell suspensions in cellulose capillary tubes. *J Microsc* 175:34–43
- Inoue T, Osatake H (1988) A new drying method of biological specimens for scanning electron microscopy: the *t*-butyl alcohol freeze-drying method. *Arch Histol Cytol* 51:53–59
- Kai J, Satoh M, Tsukidate K (1999) A new method for preparing electron microscopic specimens of *helicobacter pylori*. *Med Electron Microsc* 32:62–65
- Kaps M, Burkhardt E (1985) An improved method for electron microscopic observation of cerebrospinal fluid cells. *Acta Cytol* 29:484–486
- Mazia D, Schatten G, Sale W (1975) Adhesion of cells to surfaces coated with polylysine: applications to electron microscopy. *J Cell Biol* 66:198–200
- Morphew MK, McIntosh JR (2003) The use of filter membranes for high-pressure freezing of cell monolayers. *J Microsc* 212:21–25
- Ramandeep, Dikshit KL, Raje M (2001) Optimization of immunogold labelling TEM: an ELISA-based method for rapid and convenient simulation of processing conditions for quantitative detection of antigen. *J Histochem Cytochem* 49:355–367
- Tiedemann, Hohenberg, Kollmann (1998) High-pressure freezing of plant cells cultured in cellulose microcapillaries. *J Microsc* 189:163–171
- Van De Sandt MM, Herman CJ, Linde J (1985) Immuno- and enzyme-histo/cytochemical analysis of resin sections and cell suspensions: a comprehensive diagnosis of bone marrow on a single aspiration sample. *J Microsc* 143:205–213