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Methods in eye research

Trypan Blue staining method for quenching the autofluorescence of RPE cells for improving protein expression analysis

Girish K. Srivastava [a,b,c,*], Roberto Reinoso [a,b,c], Amar K. Singh [a], Ivan Fernandez-Bueno [a], Denise Hileeto [a], Mario Martino [a,b,c], Maria T. Garcia-Gutierrez [a,b,c], Jose M. Pigazo Merino [a], Nieves Fernández Alonso [a], Alfredo Corell [a], J. Carlos Pastor [a]

[a] Institute of Applied Ophthalmobiology (IOBA), University of Valladolid, Valladolid, Spain
[b] Castilla and Leon Regenerative Medicine and Cell Therapy Network Centre, Spain
[c] CIBER-BBN, Spain

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ABSTRACT

Retinal pigment epithelial (RPE) cells are currently in the "spotlight" of cell therapy approaches to some retinal diseases. The analysis of the expressed proteins of RPE primary cells is an essential step for many of these approaches. But the emission of autofluorescence by RPE cells produces higher background noise interference thereby creating an impediment to this analysis. Trypan Blue (TB), a routinely used counterstain, has the capacity to quench this autofluorescence, if it is used in optimized concentration. The results from the method developed in our study indicate that incubation of the cultured RPE cells with 20 μg/ml of TB after immunolabelling (post-treatment) as well as incubation of the retinal tissue specimens with same concentration before paraffin embedding, sectioning and immunolabelling (pre-treatment) can be applied to effectively quench the autofluorescence of RPE cells. Thus it can facilitate the evaluation of expressed cellular proteins in experimental as well as in pathological conditions, fulfilling the current requirement for developing a method which can serve to eliminate the autofluorescence of the cells, not only in cell cultures but also in tissues samples. This method should significantly increase the quality and value of RPE cell protein analysis, as well as other cell protein analysis performed by Flow cytometry (FC) and Immunohistochemistry (IHC) techniques.

1. Introduction

Retinal pigment epithelium (RPE) plays crucial roles in the normal function of the retina and its alterations cause some severe retinal diseases. The potential possibility to stop or delay these degenerative retinal diseases by transplanting RPE cells is considered a possible promising new therapeutic tool (Valtink and Engelmann, 2009). This approach makes RPE cells interesting for the investigators working in the field of tissue engineering, genetic engineering and cell transplantation (Majji and de Juan, 2000; Carr et al., 2009 and Wang et al., 2010). Recently numerous methods of isolation, cultivation, identification as well as transplantation have been developed. RPE cells retain their ability to proliferate in culture as well as in pathological conditions (Singhal and Vemuganti, 2005; Okada, 1980 and Stevens et al., 2005) but culturing leads to deviation from the normal characteristics of RPE cells (Saika et al., 2004). In *in vivo* conditions RPE cells retain their stability but in *in vitro* conditions they are unable to retain their characteristics such as epithelial cubical morphology, polarity, secretion of the different factors on apico-basal ends, light absorption, visual cycle of retinal, junctional barrier for metabolic transportation and phagocytosis for renovation of external segments of photoreceptors (Binder et al., 2007). This fact has motivated the investigators to utilize established RPE cell lines that do not need characterisation. But current studies have shown differences in protein expression between RPE cell lines and RPE primary cells (Alge et al., 2006 and Gupta et al., 1997), therefore, developing appropriate techniques for analysing the primary RPE cell proteins in experimental as well as in control conditions are currently required.

Currently, Flow cytometry (FC) as well as Immunohistochemistry (IHC) are techniques widely used to localize and use specific proteins to separate cells in cell cultures and tissues related studies. FC is an easy handling, quick and reliable technology for classification of the cell population according to its morphological and physiological properties (Othmer and Zepp, 1992). It identifies

* Corresponding author. Institute of Applied Ophthalmobiology (IOBA), University of Valladolid, Campus Miguel Delibes, Paseo de Belen, 17, Valladolid 47011, Spain. Tel.: +34 983 18 47 53; fax: +34 983 18 47 62.
E-mail address: girish@ioba.med.uva.es (G.K. Srivastava).

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specific cell populations but is unable to analyse the protein expression of tissue bound cells (Dent et al., 1989).

It is well known that all cells have some intrinsic level of autofluorescence, commonly caused by mitochondria, lysosomes, aromatic amino acids and other endogenous fluorophores such as pyridinic (NADPH), flavin and riboflavins coenzymes (Aubin, 1979 and Benson et al., 1979). In addition to this, RPE cells have pigments resulting in intensive emission of autofluorescence over a broad range of spectra and this causes overlaps of emission range of the spectra of commonly used fluorescent dyes. Thus, it interferes with the detection of RPE cell corresponding fluorescence over the background noise.

Trypan Blue (TB), a routinely used laboratory counterstain, has the property to quench autofluorescence (Cowen et al., 1985; Lynch and Derbyshire, 1986). In the current study, we tried to establish an FC as well as IHC method to detect specific fluorescence signals corresponding to expressed cellular proteins over cell background noise after quenching cellular autofluorescence by an optimized concentration of TB for analysing RPE cells in primary cell cultures as well as in retinal tissue.

2. Materials and methods

2.1. Reagents

Anti-RPE65 mouse monoclonal antibody (MoAb; IgG1) for detecting RPE cells was purchased from Abcam, Cambridge, UK. FITC conjugated goat anti-mouse antibody (IgG) was purchased from Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA and was used as secondary antibody. IgG from Sigma Aldrich, USA and unspecific mouse MoAbs as isotype negative controls from Beckman-Coulter, Fullerton, CA, USA were purchased.

Paraformaldehyde was purchased from Sigma Aldrich, USA. Cell Wash solution, fixation/permeabilization solution (Cytofix/Cytoperm™) and Washing solution (Perm/Wash™) were purchased from BD Biosciences, San Jose, CA, USA. PBS from Lonza, Belgium and TB counterstain solution from Lonza, Walkersville, MD USA, were purchased. The FC analysis was performed with a Cytomics FC 500 Cytometer (Beckman-Coulter, Fullerton, CA, USA) using 488 nm excitation with an argon-ion laser for FITC. The data collected were analyzed using the Cytomics RXP software program (Beckman-Coulter).

2.2. Cell culture

Pig RPE (pRPE) cells of passages P1–P6 were used in this study. pRPE cells were isolated (Dintelmann et al., 1999) from the pig's eyes gifted by local slaughter house of Valladolid, Spain and maintained in DMEM medium (Invitrogen-Gibco) supplemented with 10% foetal bovine serum (FBS), antibiotics penicillin (100 U/ml)–streptomycin (0.1 mg/ml), 1 mM sodium pyruvate, and 2 mM L-glutamine (Invitrogen-Gibco). Cells were trypsinized after cell cultures became confluent, immunostained and passed through the FC for optimizing the protocol for analysis.

2.3. Flow cytometry (FC)

Quantitative analysis of cells expressing RPE65 (Lee et al., 2010; Steindl-Kuscher et al., 2009), a specific protein marker of RPE cells, was performed to determine the deviation of cells from its natural characteristics in primary RPE cell cultures.

pRPE cells were washed with 2 ml of Cell Wash Solution and centrifuged at 500 × *g* for 5 min, the pellet was resuspended and incubated with 50 μl of IgG (40 μg/ml) for 15 min at room temperature (RT) to block Fc receptors for unspecific binding. Cells were then resuspended in 250 μl of a fixation/permeabilization solution (Cytofix/Cytoperm™) for 20 min in the dark at 4 °C to fix the cells and permeabilizate the cell membrane. After incubation, cells were washed twice with 1 ml of washing solution. Afterwards, the cells were incubated in the dark for 30 min at 4 °C with 1 μl of anti-RPE65 (IgG1) mouse MoAb. Cells were then washed with washing solution. The cells undergoing RPE65 staining were incubated in the dark for 30 min at 4 °C with 20 μl of goat anti-mouse IgG secondary antibody conjugated with FITC previously diluted 1:50 in washing solution. Cells were washed with 1 ml of 1% PBS-BSA after incubation with secondary antibody and resuspended and incubated in 500 μl (10, 20 and 40 μg/ml solution) ice-cold TB counterstain solution for 10 min at 4 °C (post-treatment). Finally the cells were washed, resuspended in 500 μl of 1× PBS and cell fluorescence was measured. Mean Fluorescence Intensity (MFI) that reflects the expression density of a given protein per cell was recorded and expressed as arbitrary units. Controls included cross-reactivity of the fluorescence signals of each channel, as well as isotype-matched unspecific MoAbs used as negative staining controls.

2.4. Retinal tissue sections (RTSs) and treatment with Trypan Blue (TB)

Pig ocular globes were collected from local slaughter house and transported in 4% p-formaldehyde in 48 h. On arrival at IOBA, the anterior segment was removed and the posterior segment of each ocular globe was cut into 3–5 mm small pieces and rehydrated by washing with PBS. Each piece was incubated with 0.5% TritonX-100 for 10 min at RT to permeabilize all the cells. Each piece was again washed and subsequently incubated with 0, 20, 200 and 250 μg/ml of counterstain TB for 15 min at RT (pre-treatment). All pieces were washed twice with PBS after incubation, dehydrated and embedded in paraffin wax. Retinal tissue sections (RTSs) of 3-μm were cut from each piece, mounted on slide with 50% glycerol under cover-slip and observed using a microscope (Leica Microsystems, Mannheim, Germany) on wave lengths, A (Blue, excitation filter (EF) BP 360/40), I3 (Green, EF BP 450–490) and N2.1 (Red, EF BP 515–560). Brightness and contrast of each photo were adjusted using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

In another set of experiments, retinal pieces were not treated with TB and were embedded in paraffin. 3-μm thick RTSs were cut from these untreated paraffin embedded pieces. Subsequently these RTSs were incubated with different concentrations of TB (post-treatment), mounted and observed using a microscope.

2.5. Immunofluorescence labelling of TB treated retinal tissue sections (TRTSs)

For RPE65 antigen immunofluorescence labelling, the RTSs (control vs TB treated) were dewaxed by immersing the slides in xylene (twice for 10 min), rehydrated with graded ethanol (5 min in 100% and 5 min in 95% ethanol), and transferred to deionised water and finally washed twice with PBS. Each RTS was pre-treated for antigen retrieval with citrate buffer, pH 6 for 15 min at 100 °C. It was followed by rinsing in water for 5 min and subsequent washing three times with PBS. And then each RTS was blocked in PBT (0.5% TritonX-100 in PBS), 10% normal goat serum (Sigma-Aldrich), and 1% BSA (Sigma-Aldrich) during 1 h at RT. The RTSs were incubated, after blocking, with anti-human RPE65 MoAb, previously diluted 1.50 in PBS, for 30 min at RT. All RTSs were washed three times in PBS and exposed for 30 min at RT to a FITC conjugated goat anti-mouse IgG secondary antibody, previously diluted 1:50 in PBS. Slides were then cover-slipped with 1:1 PBS–glycerol. Negative controls included omission of the primary antibody or secondary