Modified Protocol For Separation Of Chicks' Intestinal Epithelium From Lamina Properia Applying Flow Cytometer

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Abstract: Epithelial cells protect the surface of body cavities, and as they considered part of the immune system that fights invading bacteria. The epithelial barriers reduce the migration rate to host organs. Salmonella enteritidis is a facultative intracellular pathogen capable of surviving within non-phagocytes like epithelial cells and initiates the inflammatory responses. The current modified protocol was used for the separation of intestinal epithelial cells of newly hatched chicks from the submucosa and muscular layer that invaded by salmonella. Also, this protocol described the detaching of epithelial cells from its junction by using drop-shape steel lees stain loop and gently inverted the very small intestine of the chicks, then mechanical air pump through inverted tissue to separate epithelial cells, trypsin cells to ensure singlet status to be analysed by flow cytometer to study the invasion of microbial load and cytopathological status of epithelial cells.

Keyword: Intestinal Epithelial Cells, salmonella enteritidis.

1. Introduction

Protocol for separating murine adult intestinal epithelial cells from the underlying muscular and mesenchymal tissues typically involved extended incubations, punitive mechanical treatment, and exposure to either proteases or chelating agents. The downsides of these approaches include fragmentation, contamination with other cell types, reduced viability, and under-portrayal of crypt cells. [1], described a gentle procedure that allows harvesting of pure, fully viable, intact and contaminated-free sheets of a murine intestinal epithelium with crypts and villi from small intestine and colon were isolated in only 30 minutes on ice, without enzymes or EDTA. Previous Protocol for separation the epithelial cells from the connective tissue of the lamina propria, [2], [3], typically depending on mechanical agitation of the tissue alone or in combination with proteolytic enzymes, chelating agents, or altered osmolarity. As a result, neither epithelial nor mesenchymal preparations are free from contamination by the other cell type. Detached cells are recovered by centrifugation, which certainly results in lysis of some cells and reduces the yield. Isolation of intact epithelium requires perfusion of the animal with a high concentration of EDTA and release of the epithelium by vibration [4]. Cell Recovery Solution (or 'matrigel'), a non-enzymatic solution used to depolymerize the extracellular matrix of the basement membrane, obtaining pure epithelial preparations [5], [6]. However, that method requires extended incubations (24 hrs for adult cells) followed by shaking to release the epithelium, generating tissue fragments that are recovered by centrifugation. The current study aimed to modify Nick and Carlsson protocol to be applicable from newly hatched and chicks less than a one-week age by confirming the viability of detached epithelium cells. The procedures described here provided a rapid and efficient way to isolate epithelium and mesenchyme as the basal line for flow cytometry analysis to detect viable/apoptotic cells, detection invasion pathogens in single epithelium, gene expression analysis, and determine the cell cycle in immunopathology. The integrity of the epithelium offers possibilities for invasion parametric evaluation in population cells and pathogenic protein effectors analysis.

2. Material and Method

Induced inflammation in newly hatched chicks was done by the inoculating of Salmonella spp intercrop at dose of 10-5. The inflammatory lesions in the intestine of inoculated chicks were cut into 5cm length, rinsed several times the tissue with ice-cold phosphate-buffered saline (PBS) that was performed thoroughly to remove excess mucus and intestinal debris. Stainless steel rod (drop-shaped tip) was used to gentle

invertion of the intestine after suturing with non-absorbable surgical suture, (figure, 1). The inverted intestinal piece was submerged in a 2 ml ice-cold BD cell recovery solution (matrigel) and 1ml cold PBS in a 15 ml tube and placed on ice. Then, inflation by pushing down on the pipetted tip Air was pumped into the intestine piece using tips (100-1000µl) which was found more suitable as the intestine of chicks was very small. The pump air step was repeated 20-30 times. Finally, intestine pieces were left for 10 minutes in the air pressure state to push crypts out of the crypt beds and remove coherent sheet from the inflated intestine [1]. Matrigel solution was used to ensure the complete dissolve of basement membrane by mechanical pressure. The intestinal tissue was removed after detaching the sheet. The epithelial cells on the sheet were subjected to trypsin to make the epithelial cells free, single in suspension. Immediately, put the suspension tube in ice-cold freezers to ready preparing for Flow cytometer (BECKMAN COULTER) protocol to detect both viability and apoptosis

Figure 1: *inversion intestine by loop*

2.1 Validation of Protocol

2.1.1. Epithelial cells preparation

(a) Permeabilization of cells

The preparation was beginning in fixation and permeabilization of cells in and transferred in suspension to detect apoptosis/viable epithelial cells for flow cytometry analysis by Intracell kit (Immunostep S.L, Spain), reagents remains intact the cell surface marker expression and the cell properties of forward scatter and side scatter curve (FSC and SSC). Fifty microliters of cell suspension were pipetted to be analysed (up to 106 cells) into a tube. An appropriate volume of apoptosis detector was added from Annexin V-FITC kit (according to requirements of detection Apoptosis, Necrosis and viable cells described by manufacturer, MACS Miltenyi Biotec) directed to the cell surface antigen of interest and the appropriate isotype control. Incubation for 15 minutes in darkroom temperature was done, the step used for direct immunofluorescence staining on cell surface antigen, to detect cell membrane damage. 100 µl of Intracell Reagent a (fixative) was added to each tube, then mixed gently. Incubation for 15 minutes at room temperature was done. Washed once in 2ml PBS working solution 1X (1X phosphate-buffered saline solution, PBS +0.1 NaN3+4% BSA). Centrifugation for 5 minutes at 300 ×g, then the supernatant was aspirated, leaving approximately 50 µl of fluid. Vortex was applied to ensure the pellet in suspension. 100 µl of IntraCell Reagent B (permeabilization) was Added to the tube. The appropriate volume of the conjugated Intracellular apoptotic detector was added for the intracellular antigen. Incubation for 15 minutes in darkroom temperature was applied. Washing once in 2ml PBS working Solution 1X was done. Repeated centrifugation for 5 minutes at 300 ×g, then the supernatant was aspirated, leaving approximately 50 µl of fluid and vortex was applied to ensure that the pellet in suspension. The cell pellet was re-suspend in 0.5ml of 1% an appropriate fluid for flow cytometric use and stored in dark at 2-8C^{o}. The fixed cells were analysed for Apoptosis, Necrosis and Viability detection within 24hrs. The detection was done by staining procedure used Annexin V-FITC kit (MACS, Miltenyi Biotec), Apoptotic cells were stained positively for Annexin V-FITC that binds to phosphatidylserine (PS), while negative for staining with Propidium iodide (PI). Dead cells were stained positive for Annexin V-FITC and PI, whereas viable cells were negative for both Annexin V-FITC and PI. (Miltenyi Biotec). Epithelial cells labelled by fluorescence and suitable volume were up to 10^{-6} total cells, (Miltenyi Biotec). Cells number was determined. 10^{6-} in 1ml of 1× Binding Buffer (250µl 20× Binding Buffer Stock Solution with 4.75ml of sterile DW) was washed. Washing was repeated. Cell pellet in 100µl of 1×binding Buffer was suspended per 106 cells. 10µl of Annexin V-FITC was added per 106 cells. Mixed well and incubated for 15 minutes in darkroom temperature. Cells were washed by adding 1mL of 1X binding Buffer per 106 cells and centrifuge at 300 X g for 10 minutes. The supernatant was aspirated completely. Then washing was repeated. Cell pellet in 500µl of 1X Binding Buffer was suspending per 10^{-6} cells. Finally, 5µl of PI solution immediately added before analysis by flow cytometry.

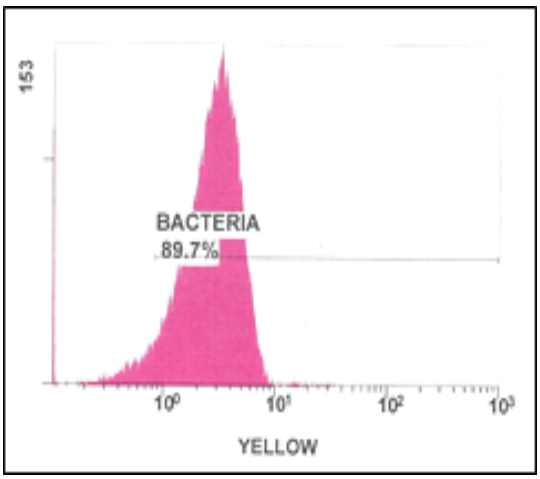

Figure 2: *One parameter histogram for counting of dead epithelial cells using PI stain.*

Table 1: *illustrate dye indicators*

Reaction		Mean
Positive	Negative	
Annexin V-FITC + PS	PI	Apoptotic Cells
Annexin V-FITC + PI		Dead Cells
	Annexin V-FITC + PI	Viable Cells

(b) Designate Protocol to Applying Analysis by Flow Cytometer

Designate histograms to analysis automatically as the following;

1- FS/AUX to select the single cells.

2- Forward scattered/side scattered (FS/SS) to select the size and granulation of epithelial cells (intact cells not destructed) I: e, whole cells.

3- Annexin V/PI to divide the cells population to the following;

I. To select live cells.

II. To select apoptotic cells.

III. To select dead apoptotic cells.

IV. To select non-apoptotic dead cells.

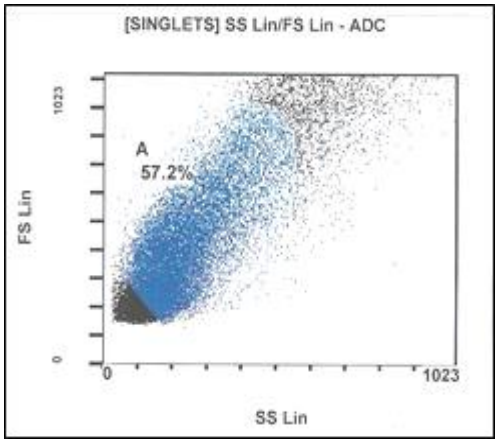

Figure 3: *This histogram showed the percentage of epithelial cells which invasive by bacteria (89.7%) within the first three hours.*

3. Result

Discussion

Dissociation of the intestine is performed by isolate of epithelium without contamination by mesenchymal cells. Nik and Carlsson state demonstrated protocol on the murine intestine, but with minor adjustments, it should be applicable from other vertebrates. The current study achieved the method of Nik and Carlsson in addition to altering stainless steel rod such as a fine steel crochet hook to thin stainless steel wire contain drop-shape on the tip and replacing the syringe tool to pipette channel with tips (100-1000µ).The advantage of the ability of BD Cell Recovery Solution to dissolve the basement membrane, and used to isolate cultured cells in Matrigel, it's not enzymatic activity and not contains EDTA but instead depolymerizes the extracellular matrix, thereby disrupting the basement membrane and its compatibility with subsequent in vitro culture is well established, [1]. The study found the compatibility of this technique and approved flow cytometric techniques. When a single cell suspension is desired, for running into a flow cytometer, [7], this is easily achieved by brief exposure to trypsin, [1]. Our study showed when using the protocol of Nik and Carlsson, 2013 after modify the method for very small intestine the Flow cytometric quantification of epithelium displayed clearly as scattered singlet cells regarding to intestinal epithelial cells and when perform gating to target epithelial within population, there was no cell lysis or contamination by cellular debris into total population. The exceptional notes to dead cells in normal features of physiological ultrastructure of the epithelium, description to programmed cell death, and cell injuries. However, the absence bias in cell cycle or pathological features in figure (annexin V Vs. PI) illustrating the agreement with validating test to a modified protocol of Nik and Carlsson.

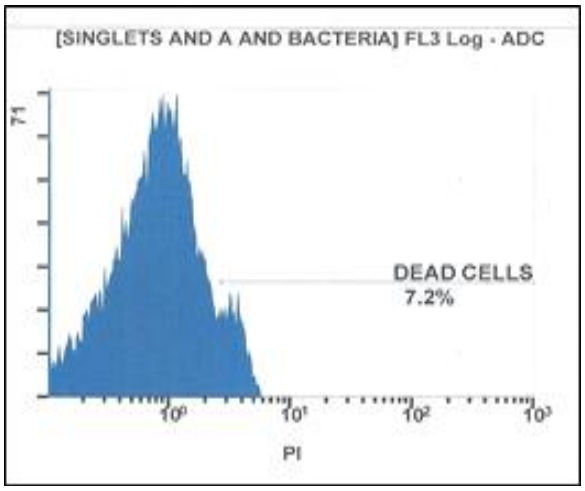

Figure 4: *FS/SS histogram for epithelial cells showed 57.2% of total epithelial cells (Blue colour) were selected for the next gating.*

4. Reference

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