



Modified SEM Preparation for THP-1 Monocytes

Heriberto DeLeon III*, Anxui Kuang, Francisco Rugama and Thomas Eubanks

Department of Biology, University of Texas Rio Grande Valley, Edinburg, Texas, USA

Abstract

THP-1 monocytes are non-adherent white blood cells that are typically differentiated into macrophage like cells to induce cellular adherence for cellular viability assays. However, the induced differentiation also alters the external cellular morphology of THP-1 cells, therefore protocol modification and development has led to a novel method of preparation of THP-1 monocytes for scanning electron microscopy. Incorporation of a polystyrene substrate, modification of the dehydration gradient and removal of critical point drying has been shown effective in preparing THP-1 monocytes for observation without alteration of external structure. Analysis of cellular integrity was subsequently via treatment with 100 nm recombinant Panton-Valentine leukocidin to induce cell lysis and allow observation of internal cellular composition and quality of preservation of the proposed preparatory protocol.

Keywords: Electron microscopy; Protocol; Specimen preparation; THP-1; Monocytes; Polystyrene substrate

Introduction

Mammalian THP-1 monocyte cultivation and preservation has been modified to produce a novel method for SEM observation. Non-adherent THP-1 cells are utilized for research applications in disease pathogenesis, drug target studies and morphological analysis. However, THP-1 cells are difficult to prepare for SEM observation using traditional preparation methods, therefore requires a new method of preservation that reduces cellular artefacts.

Cellular viability assays and effective treatment analysis in leukemia based research incorporates the application of THP-1 cells into drug treatment studies to directly observe cellular responses. However, THP-1 cells proliferate in suspension and generally do not adhere to the culture flask (or petri dish surface). Thus THP-1 differentiation is promoted to induce cellular differentiation and attachment of the cells to the culture flask, thus allowing direct observation and study of cellular response. However, these methods typically alter the morphology of monocytes to resemble macrophage like structures, thus altering the morphology of the cell surface and potentially the assertions of treatment effectiveness. It is proposed here, that the most effective applications of treatment research are to reduce variation in morphological composition and develop an accurate representation of monocyte response to potential leukemia treatment research.

The novel method presented here allows the adherence of the THP-1 cells without inducing macrophage like differentiation and preserving the monocyte status. Therefore, allowing further studies with THP-1 cells, without differentiation, centrifugation or critical point drying (all of which have been shown to affect external cellular composition). The recombinant bi-component Panton-Valentine leucocidin (PVL) model is selected for this study to exemplify leukemia based target therapies against THP-1 cells and the viability of this protocol for visual confirmation of cellular viability.

Increasing studies have now determined that the selective nature of the PVL toxin to polymorpho neutrophils, monocytes and macrophages holds a significant application in potential leukemia treatment. Therefore, the selection of the PVL model is based on two premises: first) that new emerging evidence of PVL in leukemia research applications is significant to the development of a new method of observation and visual confirmation of treatment effectiveness. Second), the PVL model has also shown that macrophage like

differentiation may be accomplished via the recombinant Panton-Valentine Leukocidin-S subunit (rLukS-PV) [1]. Where rLukS-PV has been shown to induce cellular differentiation and attachment in THP-1 monocytes and increase cellular sensitivity (*in vitro*) to the bi-component PVL toxin [2] when introduced to a cell culture prior to treatment with the complete PVL.

Thus it is the purpose of this research to develop a new method of specimen preparation for the direct observation of THP-1 cells under the scanning electron microscope with respect to monocyte target therapies (PVL working model) for visual confirmation.

Materials and Methods

THP-1 cells (ATCC, Manassas, VA, USA) were cultivated in RPMI 1640 complete (GIBCO, Darmstadt, Germany) supplemented with 10% fetal bovine serum and 1% Antibiotic Antimycotic in 75 cm² flasks at 37°C in a humidified incubator at 5% CO₂ [3].

Cells were spun down for 5 min at 300 g in a 15 mL conical centrifuge tube, then resuspended in 10 mL of fresh media. Modification of the cultivation protocol was implemented to accommodate a polystyrene substrate for specimen processing [4]. 100 mm polystyrene substrates were excised and coated with a poly-D-lysine solution (P7280-5MG) to increase cellular adherence of the suspended cells, as described by De Leon [4]. The lysine coated polystyrene disk substrates were placed in six wells of a sterilized 48 flat well microtiter plate. 1.0 mL of cell suspension (2.0 × 10⁵/mL) was pipetted into each of the six wells, without disturbing the placement of the polystyrene substrate [4]. Cells were then incubated for 72 h at 37°C with 5% CO₂.

Secondary samples were then treated with a 100 nm Panton-Valentine leukotoxin (IBT Bioservices, Gaithersburg, MD, USA) for six h to induce cell death [5]. Cells were then immediately washed in deionized water and fixed (as described previously). THP-1 cells

***Corresponding author:** Heriberto DeLeon III, Department of Biology, University of Texas Rio Grande Valley, Edinburg, Texas, USA, Tel: 832-573-5521; E-mail: heriberto.deleon01@utrgv.edu

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were also differentiated into macrophage like cells utilizing 50 μ L of the rLukS-PV sub-unit to observe morphological differentiations [1]. rLukS-PV was added to the cultivation media 36 h prior to fixation, with a subsequent media change 24 h post differentiation.

The polystyrene substrate was carefully recovered and placed on to a glass slide and observed under a light microscope to confirm cellular adhesion before processing [4]. Upon confirmation of cellular attachment, specimens were carefully placed into a 25 mm petri dish, and washed for 30 s in cold 0.1 M cacodylate buffer (pH 7.2). Samples were immediately fixed in 2.0 mL of 2.5% glutaraldehyde and 2.0% paraformaldehyde combination in 0.1 M cacodylate buffer (pH 7.2) for 30 min. Specimen samples were then post fixed with 2.0 mL of 1.0% osmium tetroxide in 0.1 M cacodylate buffer solution for 30 min and washed in molecular grade water for 3 min to remove excess osmium tetroxide.

THP-1 cells were then dehydrated in a 2.0 mL ethanol gradient series (25%, 50%, 75% and 100%) for twenty min at each step with 5 min transition gradients in between each step [4]. After the 5 min transitions, all the dehydrant (2.0 mL) was removed and replaced with the next level in the dehydration series for twenty min and repeated to 100% ethanol. The cells were then dehydrated with 50%/50%, anhydrous ethanol/acetone solution for 35 min to ensure further cellular dehydration.

Prior to full evaporation of the anhydrous ethanol and acetone, disks were removed from the anhydrous ethanol and placed on filter paper to remove excess dehydrant solution from the opposite face of the polystyrene disk (to allow proper adhesion) and placed on to a Zeiss 10 mm aluminum stubs with double sided nonconductive adhesive. While mounted on the SEM observation stub, 20 μ L of 50%/50%, anhydrous ethanol/acetone was pipetted directly on to the sample to prevent rehydration and exposure to air.

The mounted polystyrene disks did not require critical point drying and proceeded directly to sputter coating following dehydration. The samples coated with 20 μ L 50%/50%, anhydrous ethanol/acetone were placed into a Denton Vacuum Desk II Sputter Coater and pressurized for 30 min (a 20 min incubation in the sputter coater at the increased atmospheric pressure evaporated the residue anhydrous ethanol). The

samples were then sputter coated for 45 s at 45 mA less than 150 mTorr pressure to produce a gold/platinum particle coating [4]. Once sputter coated the samples were placed into a sterile desiccator cabinet for 24 h to allow a complete gradual dehydration. The prepared samples were then place into a Zeiss EVO LS10 (Oberkochen, Germany) scanning electron microscope for observation.

Results

Initial THP-1 monocytes were imaged without critical point drying 24 h after sputter coating. Samples showed preservation of cellular composition without disruption of morphology. Cellular adhesion was also evident by preservation of extracellular proteins (Figure 1) on the polystyrene substrate.

Non-treated controls (Figure 2A) showed consistent cellular morphology with cells in Figure 1. THP-1 cells treated with the PVL toxin showed significant cellular degradation (Figures 2B-2D) in response to the six hour 100 nm PVL treatment. Cellular lysis was evident post cellular attachment, indicated by the preservation of the adhesive proteins on the polystyrene substrate. Cellular fragmentation in Figure 2B shows membrane disruption and evident cytoskeletal structures. Figures 2C and 2D also shows the fixation of internal cellular protein globules within the degraded cell structures.

Macrophage like cells was differentiated from THP-1 monocytes with 50 μ L rLukS-PV (Figure 3B). External morphology shows enhanced extracellular projections, consistent with differentiated THP-1 cells. Macrophage like cells shows a rounded cellular morphology, whereas the controls show irregular rounded morphologies. Results indicate that both the treated and non-treated cells adhered to the polystyrene substrate coated with poly-D-lysine.

Discussion

Poly-D-lysine is a common cellular adhesion component of cellular cultivation flasks and petri dishes. The poly-D-lysine coating facilitates the attachment of mammalian cells to culture flasks [6], therefore it was reasoned that the optimal method would be to develop a completely removable coated substrate that would allow the direct attachment of mammalian cells during cultivation thus allowing the researcher to

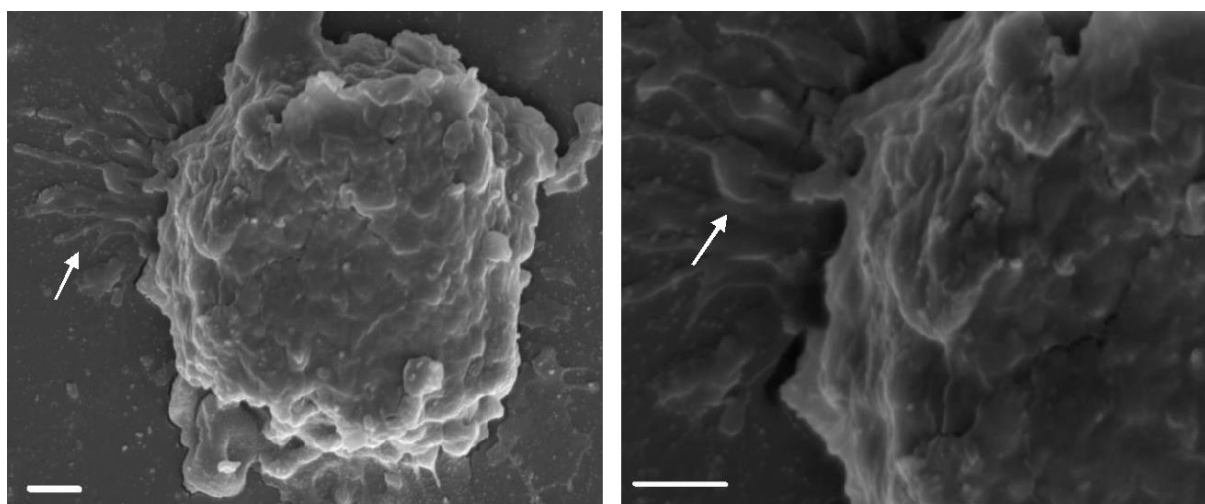


Figure 1: THP-1 Monocytes. THP-1 monocytes on a lysine coated polystyrene substrate. A) Shows an undifferentiated THP-1 monocyte after 36 h at 5.38 kV, with various area of attachment (White arrows) (Bar=1 μ m). B) Increased magnification of THP-1 monocytes emphasizing cellular morphology and attachment (White arrows) (Bar=1 μ m).

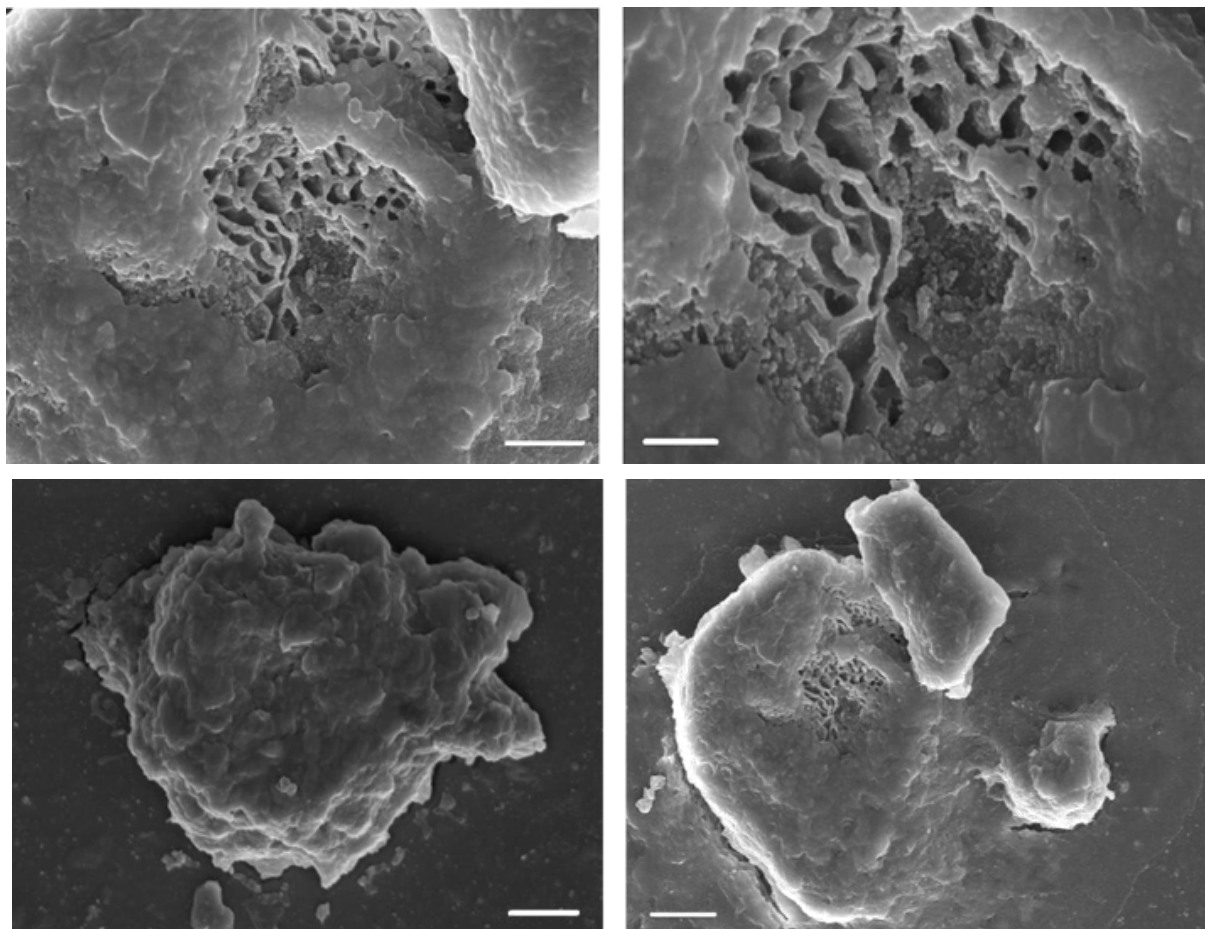


Figure 2: A) THP1 SEM Cell Lysis. A) Untreated control THP1 cell, cell remains intact and consistent with monocyte morphology (Bar=2 μ m). B) THP1 cell treated with 100 nM rPVL at 6 h, cell death induces with visible damage to cellular membrane (Bar=2 μ m). C) Increased magnification of damaged cell membrane observed in Figure B (Bar=2 μ m). D) Increased magnification of damaged cell membrane from Figure C. Internal cellular structures are visible, including globular proteins and possible cytoskeleton fragmentation (Bar=1 μ m).

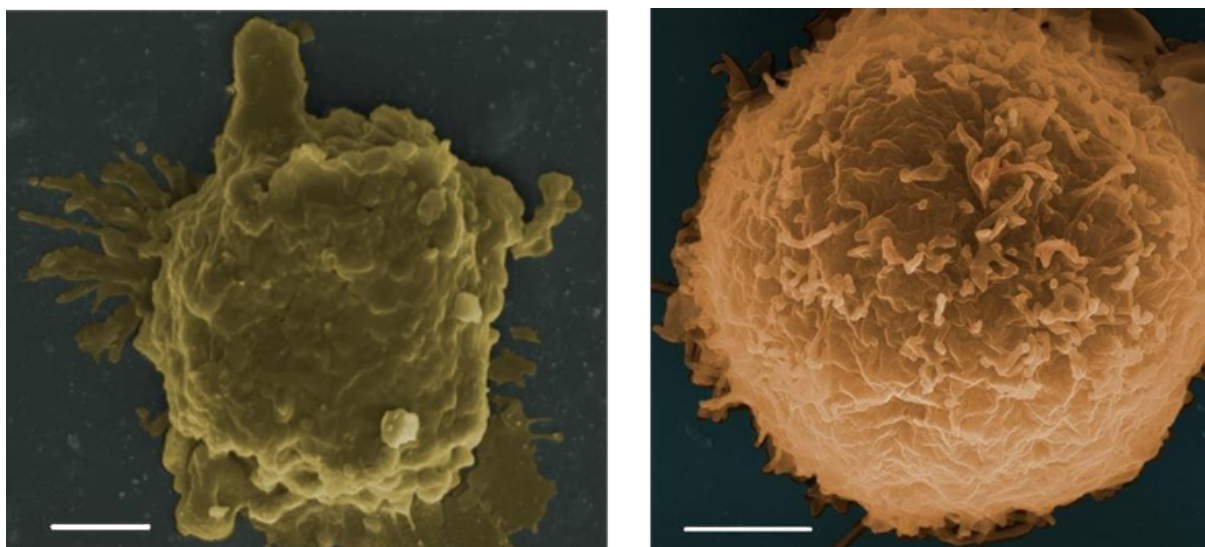


Figure 3: Scanning Electron Micrograph of THP1 Cells. A) False colored micrograph of Control THP1 cell attached to a polystyrene substrate grown in RPMI 1640. (Bar=2 μ m) B) THP1 cell treated with 50 μ L rLukS-PV, induced macrophage like differentiation with clear variation in morphology against the control.

reduce the number of transfers, centrifugations, and applications of cell releasers such as trypsin. Given the increased ability to remove the polystyrene substrate allows for a simplified handling protocol that allows the direct translocation of adhered cells to different culture wells, treatment conditions, or specimen processing applications.

The application of the poly-D-lysine coating on the polystyrene substrate allows for the THP-1 cells to be directly cultivated on the polystyrene substrate and therefore removes the need for centrifugation required to isolate non-adherent cells [5]. Removal of the centrifugation reduces the possibility of lysing pressures or mechanical stressors on the cell structure, as confirmed by Peterson et al. [7]. This allows researcher to further conclude that the alteration of morphology, if any, for the incorporated treatment was definitively from the treatment and not an artefact of specimen processing.

Furthermore, critical point drying has been generally recommended for SEM THP-1 monocyte preparation [8,9], however was removed from this protocol to reduce the probability of external morphology shrinkage or collapse. El Abed et al. [10] showed that critical point drying alters the structure of external glycoproteins, thus indicating a significant alteration to the cells adhesive capability. Therefore, development of a SEM preparation protocol that reduced the dependence on critical point drying was considered key to simplification.

Finally, preparatory methods described by Osier et al. [8] had a limiting accelerating voltage of 2kV, where samples prepared with the novel method presented here peaked at 13 kV without shifting or vibration indicating successful dehydration and the potential to handle higher levels of voltage when observing specimens. This may allow for higher resolution of the THP-1 cells, indicating the quality of the fixation and dehydration method presented here. Cells prepared here were consistent with those in publication [5,8,9], therefore indicating the novel method presented here as a viable alternative to currently published protocols.

Conclusion

THP-1 monocytes are primarily utilized in leukaemia research to study the effects of target drug therapies, disease progression, and general immune function. Therefore, the novel method of SEM sample preparation to promote adherent, non-differentiated THP-1 monocytes

without centrifugation or critical point drying is vital to provide the most accurate visual confirmation of target efficacy. Utilizing the PVL working model, this study highlights the potential for direct observation of treatment interactions on THP-1 cellular viability and integrity, while reducing variation in cellular composition.

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