# Cell membrane rupture: A novel test reveals significant variations among different brands of tissue culture flasks

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**Research note**

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# Cell membrane rupture: A novel test reveals significant variations among different brands of tissue culture flasks

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## Abstract:

Objectives: Loss of cytoplasmic molecules including protein controls, due to cell membrane rupture can cause errors and irreproducibility in research data. Previous results have shown that during the washing of a monolayer of cells with a balanced salt solution, the fluid force causes cell membrane rupture on some areas of the flasks/dishes. This fact shows the non-uniformity of the polystyrene surface in terms of cell culture. There is at present no simple test to monitor that surface. This paper presents a novel biologically based assay to determine the degree of heterogeneity of flasks supplied by various manufacturers.

Results: This paper shows that significant variation exists in polystyrene surface heterogeneity among several brands of tissue culture flasks, varying from 4% to 20% of the flask surface. There is also large variability within the production lot of a manufacturer. The assay method involves loading the cells with a cytoplasmic fluorescent marker that is released upon cell membrane rupture. Cell membrane rupture also causes the loss of marker proteins such as GAPDH used in Westernblots. This novel assay method can be used to monitor the batch consistency and the manufacturing process of flasks/dishes. It may also be used to test new biomaterials.

Key words: polystyrene, tissue culture, cell membrane stability

## Introduction:

The behavior and functions of cells in-vitro are affected by the physical microenvironment including the substratum [1, 2]. For example, the substratum strain and rigidity affects cell motility [3], and fluid shear forces upregulate signal transduction processes in endothelial cells [4].

Polystyrene (PS) is the material used ubiquitously in cell culture [5]. We have reported that when a fluid force, such as the rinsing of cells after the removal of medium, is applied to the monolayer, on certain areas of the flask or dishes, the plasma membrane of adherent cells will rupture immediately while the cells are still adherent to the PS surface [6]. This effect appears to be related to the stress in the PS polymer that developed during the manufacturing process and can be demonstrated with birefringence pattern in the plastic [7]. This fluid shear effect was initially observed with the squamous cells line, NBT-II and several epithelial cell lines. Some epithelial cells lines do not respond to fluid shear. For example, MDCK cells that have tight junctions do not respond. Breast cell line MCF10A responds to fluid shear similarly to NBT-II cells but MDA MB231 cell line does not respond to fluid shear. For comparison of these two breast cell lines, please see additional file #1.

For molecular and biochemical analysis of the cultured cells, after the removal of the culture medium, cells are generally rinsed with a buffered salt solution such as PBS or HBSS. Therefore, a concern is that the rinsing process can cause the loss of house-keeping marker proteins such as GAPDH used in Westernblots. Specific target molecules in the cytoplasm may also be lost, leading to variability in repeating experimental results, as well as the irreproducibility of the results from another lab that uses different flasks/dishes.

Variability in PS surface has been demonstrated in PS flasks and dishes by physical measurements [8]. This paper describes a novel biologically based method to determine quantitatively the percent of the heterogeneity of the PS surface in tissue culture flasks and dishes that contribute to cell membrane rupture due to fluid shear force. This novel method can be helpful for the end user to compare the consistency of batches of plastic ware. This method can also be used by PS manufacturers to monitor the quality of their production, including any changes in the manufacturing process and for testing new biomaterials.

# Main Text

## Methods and Materials:

Samples of T-25 flasks were obtained from selected manufacturers (Chemglass, Corning, Falcon, Greiner, Nunc, Santacruz, Sarstedt, TPP).

Details of methods and material is presented in additional file #2

**Protocol of fluid shear in flasks:** The detail of protocol is presented in additional file #3. Briefly, cells grown in T25 flasks from various manufacturers were incubated with 2μM CalceinAM for 60 minutes. After first rinsing with cold HBSS, then warm HBSS to apply fluid shear, the supernatants are collected for fluorescence measurement. The flask is then incubated with TritonX100 to release the remaining fluorescence in cells. The percentage of fluorescence released by fluid shear to the total fluorescence is calculated and expressed as the percent of heterogeneity of the flask surface.

## Results:

Initially it was thought that the release of housekeeping proteins such as GAPDH used in WesternBlots could be used to quantify the fluid shear effect. However, qualitative fluorescence imaging of Alexa488 labelled antiGAPDH showed that after fluid shear, there is some loss of the marker protein from the cytoplasm in some cells, but in other cells, GAPDH may be retained to varying degree in the nucleus of the cells as shown in figure 1.

**Figure 1:** GAPDH and PI staining in Fluid Sheared NBT-II cells .

**Figure 1 legend:** Cells were preloaded with CalceinAM to show green Calcein fluorescence. After fluid shear, the cells were stained with propidium iodide, then fixed and stained with Alexa488 conjugated anti GAPDH antibody. Bar at left bottom = 10μm

Since not all cells lose the marker proteins GAPDH equally throughout the culture, and GAPDH may be retained in the nucleus, GAPDH cannot be used to quantify the effect of fluid shear. Therefore, a smaller molecule that is not bound inside the cells will be a better choice to quantify the fluid shear effect.

CalceinAM, a non-fluorescent molecule is easily taken by live cells, and inside the cell, CalceinAM is converted by non-specific esterases into the fluorescent molecule Calcein [9].

Preliminary experiments show qualitatively that CalceinAM loaded cells show green fluorescence, and upon fluid shear, the ruptured cells lose the Calcein green fluorescence, and the nucleus can be stained with propidium iodide as shown in figure 2. The staining of live cells with Calcein and dead cells with propidium iodide is the basis of the Live/Dead Assay® developed by Invitrogen.

**Figure 2:** Calcein Fluorescence in NBT-II cells Before and After Fluid Shear

**Figure 2 legend:** Panel A shows cells preloaded with CalceinAM, giving green fluorecence of Calcein in the cytoplasm Panel B shows fluid shear on the cells causing some cells to lose the green cytoplasmic fluorescence (marked by star). The membrane ruptured cells appear as "ghost" cells inter-dispersed among live cells. Occasionally there are large patches of ruptured cells as shown in the upper inset in Panel B. Ruptured cells can be stained with propidium iodide as shown in the lower inset in Panel B. Bar = 10μm

As illustrated in Figure 2 panel B inset, the close juxtaposition of live and ruptured cells suggests that the heterogeneity of polystyrene is at the micro or nano level. This presents a challenge to the detection of polystyrene surface irregularity by physical methods.

Calcein uptake by NBT-II cells is dependent on cell number and concentrations of CalceinAM. There is a linear relationship of uptake between time and normalized cell number. Please see Figure 3 in additional file #4 for the kinetics of CalceinAM uptake.

Triton X100, 0.1% is used to extract total fluorescence in cells. The data showing 100% efficacy of Triton extraction are presented in additional file #4.

A comparison of T-25 flasks from various manufacturers is shown in table1a.

**Table 1a:** Fluid shear effect of NBT-II cells grown in T-25 of various manufacturers.

**Table 1a legend:** The results shown in table 1a represent 3 separate runs on each type of T-25 flasks. Therefore, the sem represent n=3. For each run, 3-4 flasks of each brand are used.
* indicates that fluid shear effect in flask is significantly greater ($p < 0.05$) than the non-shear value

The results show that there is considerable difference, varying from 4.5% to 20.7% of the total T-25 flask surface among the manufacturers. A specialized treatment to alter the PS surface charges (CellBind®) did not prevent the fluid shear effect. In comparison to NBT-II cells, MDCK cells do not exhibit fluid shear effect as shown in table 1b.

**Table 1b:** Fluid shear effect of MDCK cells grown in T-25 flasks

**Table 1b legend:** 5 Flasks of each manufacturer are used. The fluids shear effect is not statistically significant

**Discussion :**

It is a long and generally held view by researchers that for tissue culture studies, one should not change the manufacturer of tissue culture flasks and dishes lest it might affect the reproducibility of the results. This means that during manufacturing of tissue culture flasks, some factor or factors can

119 introduce heterogeneity onto the surface, causing difference among the manufacturers during the
120 injection molding of the plastic flasks or dishes.

121 To date, there is not a simple method to determine the heterogeneity of PS surface. This paper
122 describes a novel biological assay based on the fluid shear effect on adherent cells to determine the
123 heterogeneity of the PS surface used in tissue culture. As cells rupture, cytoplasmic content will be
124 released. Therefore, the potential loss of molecules of interest, such as signal transduction proteins, may
125 vary when different PS dishes are used, and can lead to irreproducibility among experiments. Indeed,
126 this paper shows that the amount of the fluid shear rupture of cell membrane is variable among various
127 manufacturers of the culture flasks, representing a potential variable when the investigator switches to a
128 different manufacturer for PS flasks and dishes.

129 Reproducibility of research data is essential, particularly in pre-clinical research. A recent paper
130 by Freedman et al. [10] shows that the cumulative cost of irreproducible preclinical research to be $28
131 billion in the US alone. The paper further showed that biological reagents and reference material
132 accounted for 36.1% of the total cost. Tissue culture technique is used widely in preclinical research.
133 However, standardization in the PS used in flasks and dishes does not exist. It is provocative to suggest
134 that owing to different amounts of cell rupture under fluid shear, variable results may occur among
135 laboratories in the analysis of cytoplasmic small molecules. Battiston et al. [11] have shown that tissue
136 culture polystyrene from different manufacturers differ considerably in protein binding and the release
137 of cytokines by monocytes interacting with the polystyrene.

138 The mechanism of cell response to the physical and chemicals nature of the substratum is
139 complex [12]. Cell functions such as motility and differentiation are influenced by the cell sensing the
140 mechanical properties of the substratum such as rigidity and smoothness [13]. The exact mechanism
141 involved in cell membrane rupture upon fluid shear on certain areas of the PS surface is not known.

However, it is expected that cell adhesion proteins such as cadherins, integrins and focal adhesion kinases may be involved [3]. The differential response to fluid shear effect by different epithelial breast cancer cell lines may cause an unreliable comparison of cytoplasmic content between two cell lines. (Please see additional file #1)

The results shown in Table 1a included the Corning CellBind flasks [14] that exhibited reduced fluid shear effect but did not eliminate the effect suggesting that PS surface charge may not be the only factor causing the heterogeneity in the PS surface. The TPP brand showed much less effect of fluid shear effect. However, the variations among the lot tested were very large.

Figure 2 Panel B illustrated the close juxtaposition of live and dead cells, suggesting that the sensing of PS surface by cells may be at micro-meter or nano-meter level. Previous studies have shown that in the edges of the flasks and dishes, and in areas related to the injection port, there is strong birefringence under cross-polarized light, suggesting that PS molecular orientation may contribute to the sensitivity of cells to fluid shear. It has been shown that during the injection mold process, as the PS polymer solidifies, shrinkage can affect the dimension of the final product but also has a large effect on the residual stress distribution of the product [15], and can be predicted [16]. Indeed, application of stress force to a disc cast from a polystyrene solution showed birefringence and cells grown on such surface demonstrated the fluid shear effect [7]. We speculate that the molecular orientation of the surface of the PS polymer is similar to that of the bulk PS that shows birefringence. Therefore, the fluid shear effect of cells may be due to cell interaction with the molecular orientation of the PS polymer at the surface. The liquid crystal alignment of a polymer surface can be modified by rubbing [17], and the resulting molecular orientation of the PS surface has been studied [18]. It will be interesting to study the fluid shear on the modified PS surface.

The biocompatibility or biological responses of biomaterial needs to be evaluated with various parameters [19]. The most common aspect of biological evaluation of biomaterials is cytotoxicity, with occasional reference to cell growth and cell adhesion. The cell line most used is a fibroblast. Our observation of the fluid shear on cell membrane represents a unique aspect of interaction between the substratum and adherent epithelial cells that may yield different aspects of biomaterial/cell interaction in the development of biomaterial.

The integrity of cell membrane under fluid shear may have relevance in vivo. In vascular physiology, under laminal flow, the EC produces anti-inflammatory molecules whereas under disturbed flow (turbulent flow) such as at arterial bifurcation and branch points, the EC can produce pro-inflammatory molecules leading to a propensity for atherogenesis at these sites [20]. Therefore, any pathological changes in the basement membrane that causes endothelial cell death in response to fluid shear could enhance the pro-inflammatory reaction. In cancer metastasis, tumor cells have been shown not to survive the hemodynamic shear force [21]. Therefore, adherence to the endothelium may alter the tumor cell response to fluid shear force.

**Conclusion and Significance:**

This paper describes a novel biologically based test for tissue culture flasks and dishes. As a cell culture is routinely rinsed before biochemical analysis, some cells will rupture, releasing cytoplasmic content including marker proteins used in Westernblots. Therefore, errors in research data can occur. Products from various manufacturers differ significantly in their surface heterogeneity. This can lead to irreproducibility of data among research laboratories.

## Limitations

The mechanism of fluid shear on cells is not known. Because the appearance of birefringence of the flasks and the response of NBT-II cells to stressed polystyrene, we speculated that this fluid shear effect of cell rupture is due to the molecular orientation of the polystyrene polymer. We have shown previously that NBT-II cells on collagen do not show fluid shear effect. In order to quantify the release of cytoplasmic marker proteins from shear cells, ELISA assay for the specific proteins would need to be performed.

**List of abbreviations:** DMSO: Dimethylsuphoxide; HBSS: Hanks balanced salt solution; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; MDCK: Madin Darby Canine kidney; NBT-II: Nara bladder tumor II; PBS: Phosphate buffered saline; PI: propidium iodide; PS: Polystyrene.

## Declarations:

Ethical approval: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The raw data required to reproduce these findings are available to download from:https://drive.google.com/drive/u/0/folders/13uj_LdCt4JQD0HTs06S6P2959Dw_pOn5

Competing interest: The author declares that he has no competing interests

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Author's contributions; No co-authors

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Figure 1 GAPDH and PI staining in fluid sheared NBT-II cells

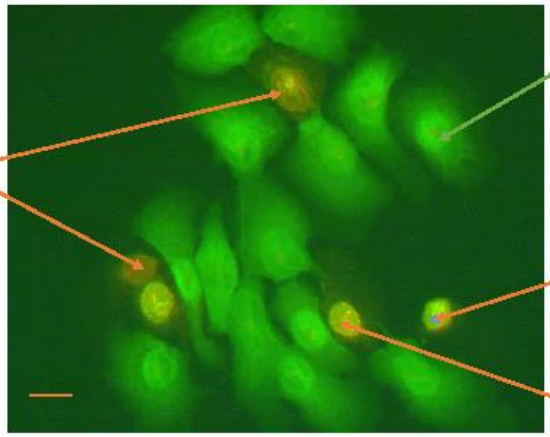


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Figure 2 Calcein fluorescence in NBT-II cells before and after fluid shear

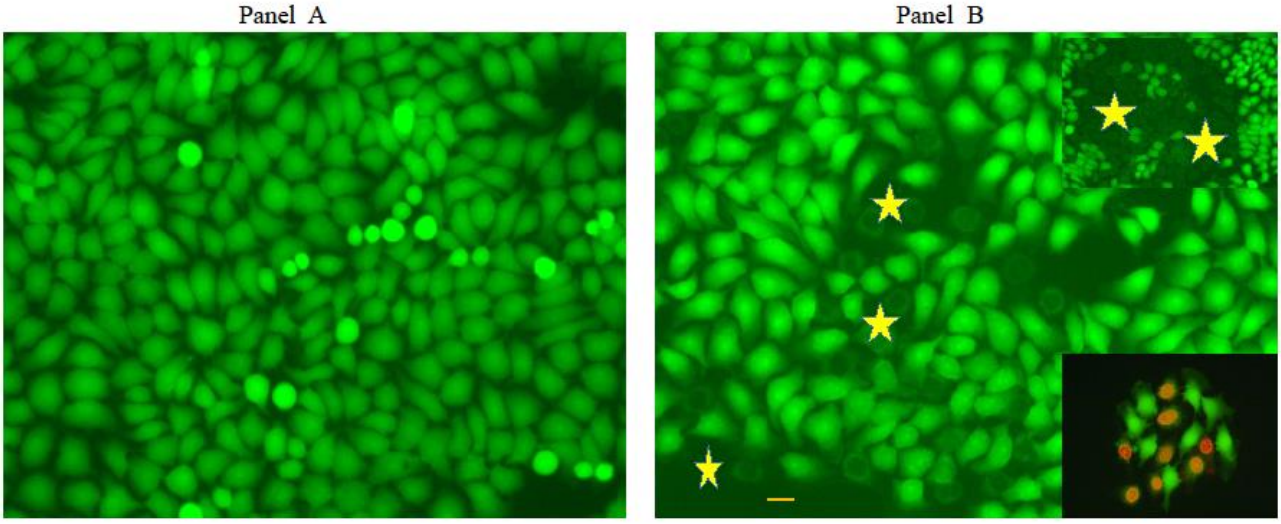


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Table 1a Fluid shear effect of NBT-II cells grown in T-25 of various manufacturers

| Manufacturer | Cat # | Lot # | % Fluid Shear Effect +/- sem |
|---|---|---|---|
| Chemglass(CellTreat) | 229331 | 110723-218 | *10.5 +/- 0.5 |
| Corning (regular) | 3056 | 09815045 | *14.1 +/- 0.6 |
| Corning (Cell-Bind) | 3289 | 22005005 | 8.7 +/- 0.5 |
| Falcon | 353108 | 5249004 | *13.6 +/- 1.4 |
| Greiner | 690170 | 08050140 | *11.3 +/- 0.2 |
| Nunc | 136196 | 136985 | *20.6 +/- 0.3 |
| SantaCruz | SC-200262 | 110423-218 | *11.8 +/- 0.5 |
| Sarstedt | 831810 | 6065081 | *13.4 +/- 1.1 |
| TPP | 90025 | 20150141 | 4.5 +/- 1.3 |

Table 1b Fluid shear effect of MDCK cells grown in T-25 flasks

| Manufacturer | % shear +/- sd |
|---|---|
| Corning | 2.4 +/- 0.4 |
| Greiner | 3.3 +/- 0.4 |

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# Figures

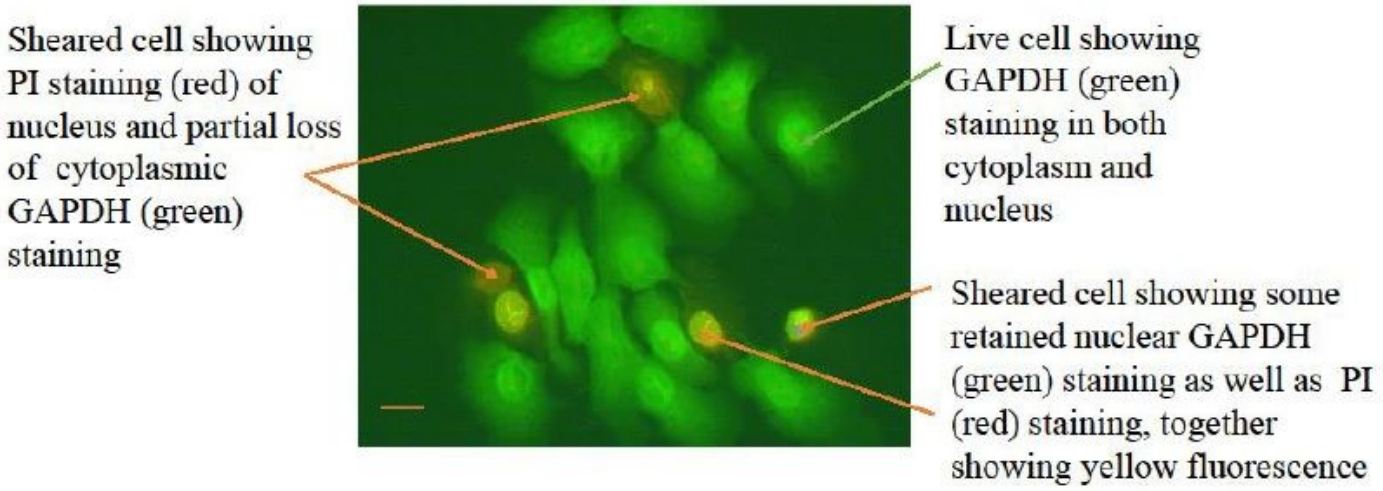


**Figure 1**

GAPDH and PI staining in fluid sheared NBT-II cells

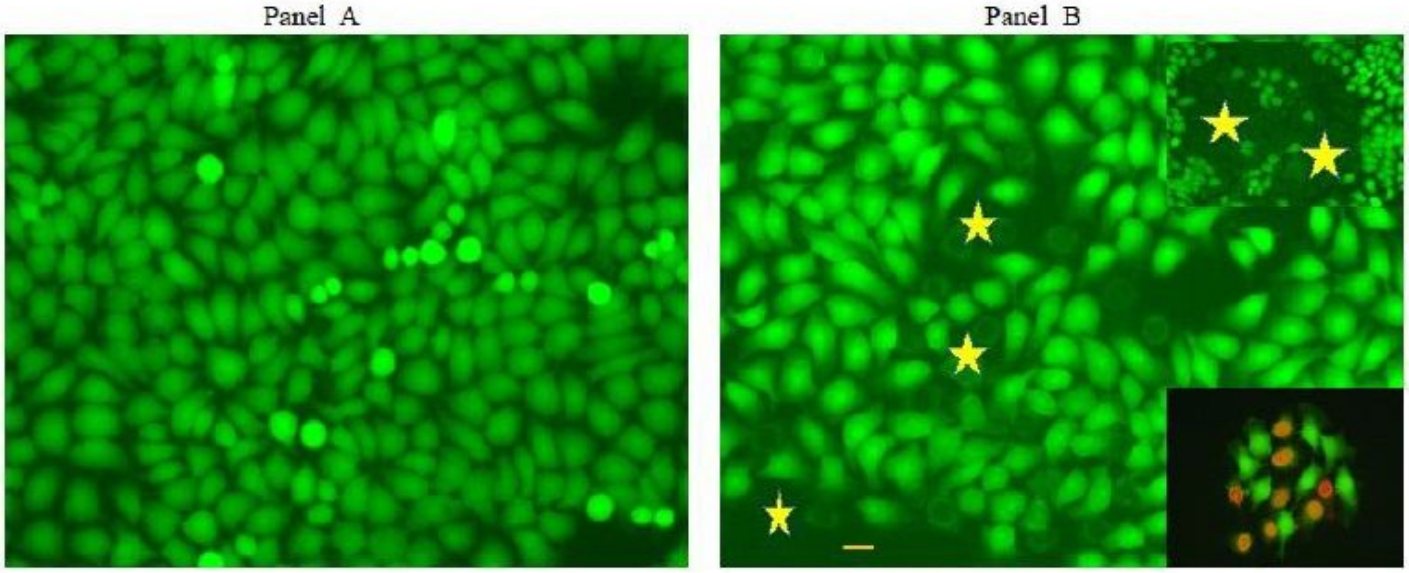


**Figure 2**

Calcein fluorescence in NBT-II cells before and after fluid shear

# Supplementary Files

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- Additionalfiles.pdf