



Autoimmune:

LYMPHOCYTE - INFLAMMATION - ADHESION ASSAY

INTRODUCTION

Cellix Ltd. has developed a novel Microfluidic Platform consisting of a PC-controlled Nanopump with microfluidic biochips and DucoCell analysis software. The Nanopump enables very accurate flow rates to be achieved which are more reproducible and consistent compared to anything currently available. Importantly, flow rates are extremely low ($5 \mu\text{L min}^{-1}$ to $10 \mu\text{L min}^{-1}$) and the shear stress levels that the pump can mimic (up to 30 dynes cm^{-2}) are equivalent to those found in blood vessels *in vivo*. The Vena8 biochips are comprised of eight microcapillaries, and are manufactured such that the dimensions of the capillaries are similar to the blood vessels being assessed. Currently, the capillaries may be coated with recombinant human adhesion proteins for use in inflammation studies. The Nanopump is vital to the use of small diameter capillaries as standard syringe pumps are incapable of delivering the required low flow rates.

OVERVIEW

First of all, the cell type to be analysed must be determined. This is followed by establishing how to harvest such cells e.g. culturing in growth media, or isolation from *in vivo* fluids. Secondly, the assay itself should be outlined, including what proteins will be used to coat the capillaries of the biochip. Thirdly, the adhesion profile of the cells to be passed through the coated capillary should be determined. Next, if exogenous compounds are being analysed, these should then be introduced to the system and their effect on the adhesion profile assessed. This should include calculation of required concentrations and pre-incubation conditions, before introduction to the system. Finally, the images taken via the digital camera attached to the microscope should be masked and analysed using the DucoCell software.

ASSAY DEVELOPMENT STEPS

i) Choice of cell type and harvesting protocols

A microfluidic assay assessing the adhesion profiles of peripheral blood lymphocytes (PBLs) was developed. Human PBLs were obtained from blood donated by normal volunteers who were not taking any medication, and who gave informed consent.

PBLs were purified by a standard technique using a T-cell enrichment cocktail (RosetteSep) and centrifugation on Lymphoprep™ gradients (see PBL isolation protocol).

ii) Assay outline, including Vena8 biochip coating procedures

Each microcapillary was coated for one hour in humid conditions at ambient temperature with either rhVCAM-1 or BSA (both $10 \mu\text{g mL}^{-1}$ in HBSS containing Ca^{2+} and Mg^{2+}). Coating with rhICAM-1 takes up to 3 hours at ambient temperature. Alternatively, all coating proteins can be applied overnight at 4°C , again in a humidified chamber. All capillaries were then coated with BSA to occupy non-specific binding sites.

The population of isolated PBLs was split. Some were stimulated with the phorbol ester PMA for 2-3 hours, while the other group was unstimulated. The adhesion profiles of non-stimulated PBLs were recorded, with respect to VCAM-1, ICAM-1, and BSA.

iii) Adhesion profiles

PBLs were infused into the capillaries at stepwise increases in shear stress, from 0 - 5 dynes cm^{-2} , one minute per shear stress level; finally increasing the shear stress to 10 dynes cm^{-2} for one minute. Images at each shear stress level were captured using the accompanying PixeLINK microscopy software. Adhesion was evaluated by monitoring both non-stimulated and PMA stimulated migratory behaviour in real time with images captured via a digital camera connected to the microscope.

iv) Image analysis

Three images per shear stress level were captured (see figure 1 for examples), and adhered PBL numbers were recorded using DucoCell application software. Data was exported into Excel for interpretation.

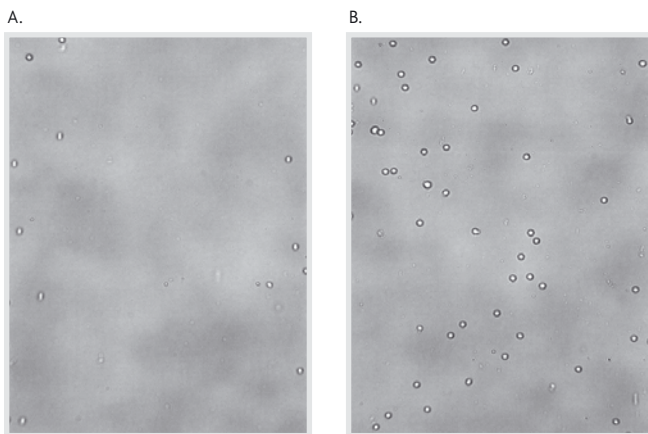


Figure 1: Microscope images (20x magnification) showing resting PBL adhesion to a) BSA ($10 \mu\text{g ml}^{-1}$), and b) rhVCAM-1 ($10 \mu\text{g ml}^{-1}$) at 1 dyne cm^{-2} . Cells were pre-incubated in RPMI with 10% FCS for 30 mins at 37°C before being passed through the biochip capillary ($400 \mu\text{m}$ wide, $100 \mu\text{m}$ deep).

RESULTS

The adhesion profiles of non-stimulated and PMA-stimulated PBLs were recorded with respect to VCAM-1, ICAM-1, and BSA (see Figures 1, 2 and 3). Distinct morphological changes in PMA-stimulated PBLs, compared to non-stimulated cells, were observed. These include a less rounded appearance of the PMA-stimulated PBLs, and evidence of "uropod" formation, which anchor the T cell to the endothelium as part of the migration process.

As expected, PBLs have very low adhesion levels to BSA. Levels of adhesion to VCAM-1 are high, but there appears to be little difference between the adhesion profiles of non-stimulated and PMA-stimulated PBLs. However, there is a distinct difference between the adhesion levels of non-stimulated and PMA-stimulated PBLs to ICAM-1, with PMA-stimulated cells showing a higher affinity for ICAM-1 than non-stimulated cells (Figure 3).

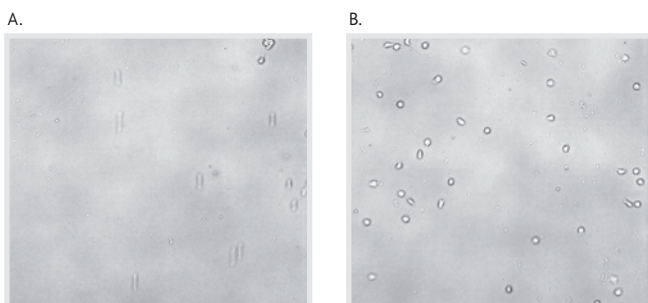


Figure 2: Microscope images (20x magnification) showing PMA-stimulated PBL adhesion to a) BSA ($10 \mu\text{g ml}^{-1}$), and b) rhICAM-1 ($10 \mu\text{g ml}^{-1}$) at 1 dyne cm^{-2} . Cells were pre-incubated with PMA in RPMI with 10% FCS for 3 hours at 37°C before being passed through the biochip capillary ($400 \mu\text{m}$ wide, $100 \mu\text{m}$ deep).

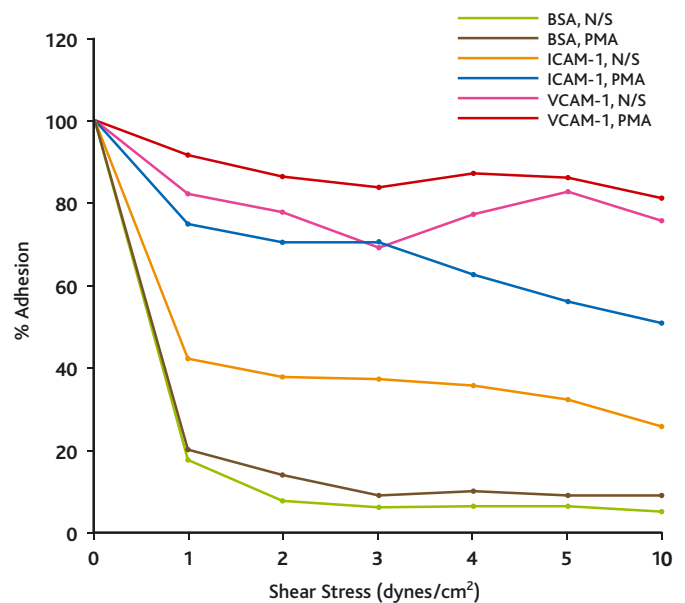


Figure 3: Non-stimulated and PMA-stimulated PBL adhesion to VCAM-1, ICAM-1, and BSA. Data presented as a percentage of adhesion at 0 dyne cm^{-2} .

ASSAY CONDITIONS

The following solutions were used during the above procedure:

PBL Wash Buffer

1x PBS
2% FCS

PBL Incubation Media

RPMI media
10% FCS
100U penicillin/0.1mg streptomycin
2mM L-glutamine

PBL Adhesion Media

CO₂-independent media
10% FCS
100U penicillin/0.1mg streptomycin
2mM L-glutamine

Note: Incubate PBLs at $5 \times 10^6 \text{ ml}^{-1}$ in the adhesion media for at least 30 mins before commencing the assay. Also, dissolve PBL stimulants/inhibitors in this media.

REFERENCES

Long, A., Mitchell, S., Kashanin, D., Williams, V., Prina Mello, A., Shvets, I.V., Kelleher, D., & Volkov, Y. (2004). A multidisciplinary approach to the study of T cell migration. *Ann. N.Y. Acad. Sci.*, **1028**, 313-319.

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