

INTEGRATED LABELLING, DISSOCIATION, ELECTROKINETIC TRANSPORT AND DETECTION OF PRIMARY TUMOUR CELLS

Jane Woods,¹ Peter T. Docker,¹ Charlotte E. Dyer,² Stephen. J. Haswell¹ and John Greenman*²

¹Department of Chemistry, University of Hull, UK, ²Centre for Biomedical Research, University of Hull, UK

ABSTRACT

Microfluidics, an enabling and emerging technology in cancer metastasis research, has yet to be applied in the analysis of individual cells taken directly from patient carcinomas. Accordingly, we have developed an integrated microfluidic methodology to label, dissociate, transport and detect patient-derived tumour cells. Following on-chip cold perfusion of tissue with antibody and enzymes, fluorescently-labelled cells were liberated and electrokinetically transported in a HEPES / sucrose agarose gel for downstream detection. Observed electrokinetic mobilities comprised dominant anodic electroosmotic flow (EOF) and opposing cathodic cell electrophoresis.

KEYWORDS: Microfluidic, metastasis, electroosmotic flow, cell electrophoresis, microflow cytometry, μ FACS.

INTRODUCTION

Metastasis research often employs flow cytometric analysis of cultured cell lines. However, results cannot be extrapolated to explain *in vivo* tumour behaviour; even 3D co-cultures do not represent tissue complexity in which multiple cell populations interact with one another and with the extracellular matrix (ECM). An ideal starting point for flow cytometric assays would be patient biopsies, but conventional cell dissociation and labelling protocols involve multiple steps, each necessitating cell washing and centrifugation leading to cell losses and the potential introduction of methodological artefacts. Microfluidics lends itself to the integration of preanalytical sample processing, and furthermore electrokinetically-driven systems have the advantages of few moving parts and simple macro-to-micro interfacing. Early work on EOF cytometry explored the manipulation of bacteria, yeast and red blood cells [1]. Subsequently, bacteria have been stained on-chip with fluorescent dyes prior to EOF-enabled sorting [2], but for human cancer cell manipulation the convenience of EOF has yet to be exploited. We present here an integrated methodology that allows antibody-labelling of cells prior to dissociation from the tumour mass, followed by electrokinetic cell transport and detection (Figure 1).

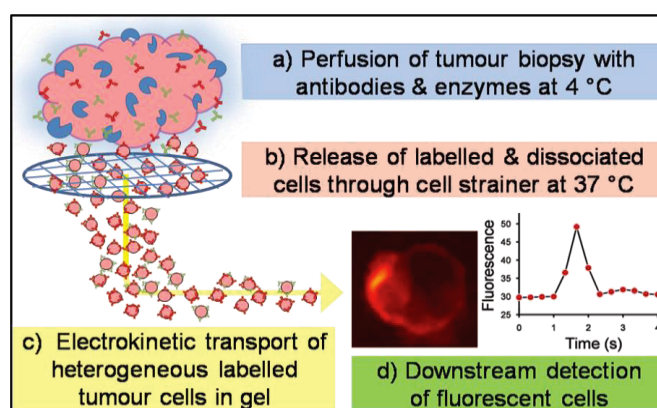


Figure 1: Schematic of integrated microfluidic processes from biopsy to fluorescent cell detection.

THEORY

Diffusion time is governed by Fick's law:

$$t = \frac{x^2}{2D} \quad (1)$$

where t is time, x is distance, and D is diffusion coefficient. For IgG₁ and collagenase in tumour tissue, D is typically $10 \mu\text{m}^2 \text{s}^{-1}$, and theoretical diffusion time to the centre of 1mm^3 of tumour tissue at $20 \text{ }^\circ\text{C}$ is $\approx 3.5 \text{ h}$. However, the effective rate of antibody diffusion is also influenced by antibody-antigen binding, decreasing inversely with antigen density [3]. Collagenase activity at $4 \text{ }^\circ\text{C}$ is relatively low; incubation at $4 \text{ }^\circ\text{C}$ for 24 h therefore preserves the tissue, enzymes and antibodies whilst permitting extensive perfusion of the ECM with enzymes and saturation of antibody-binding.

Apparent cellular electrokinetic velocity, v_{app} , is governed by the equation:

$$v_{app} = \frac{\varepsilon \cdot (\zeta_w - \zeta_c) \cdot E}{\eta} \quad (2)$$

where ε is fluid dielectric constant, ζ_w zeta potential in the electric double layer (EDL) at the microchannel-fluid interface,

ζ_c cellular zeta potential, η viscosity, and E electric field strength [4]. Cells in an electrolyte solution possess their own EDL; the negatively-charged membrane attracts a “screening cloud” of cations, causing cathodic electrophoretic flow (EPF) [4, 5, 6]. Cellular μ_{app} equates empirically to v_{app} divided by E , and comprises both EOF bulk flow and EPF. Zwitterionic buffers increase ϵ with no concomitant increase in conductivity, thereby enhancing electrokinesis [7]. Similarly, low ionic strength solutions favour electrokinesis; EDL thickness is inversely proportional to square root of ionic strength, a requirement that precludes the conventional use of salts for cell isotonicity [5]. A further issue is buffer temperature sensitivity because of Joule heating. HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] is a zwitterionic Good’s buffer with an apparent pK_a of 7.4 equivalent to physiological pH , and a low temperature coefficient (dpK_a / dT) of $-0.014 / ^\circ C$ [8]. Consequently, a low ionic strength HEPES / sucrose mixture was selected for electrokinetic cell manipulation, and used as agarose gel both to provide some resistance against hydrodynamic back flow, and also for its physical similarity to ECM.

EXPERIMENTAL

Glass microfluidic devices were fabricated using standard photolithography and wet etching techniques to produce the design shown in Figure 2. Channels $70 \mu m$ deep and $150 \mu m$ wide were etched in 1 mm borofloat glass. A 3 mm diameter tissue chamber and 1.5 mm diameter holes for sample, reagent and electrode access were drilled in a 3 mm glass top plate before thermally bonding it to the etched base plate to form the device.

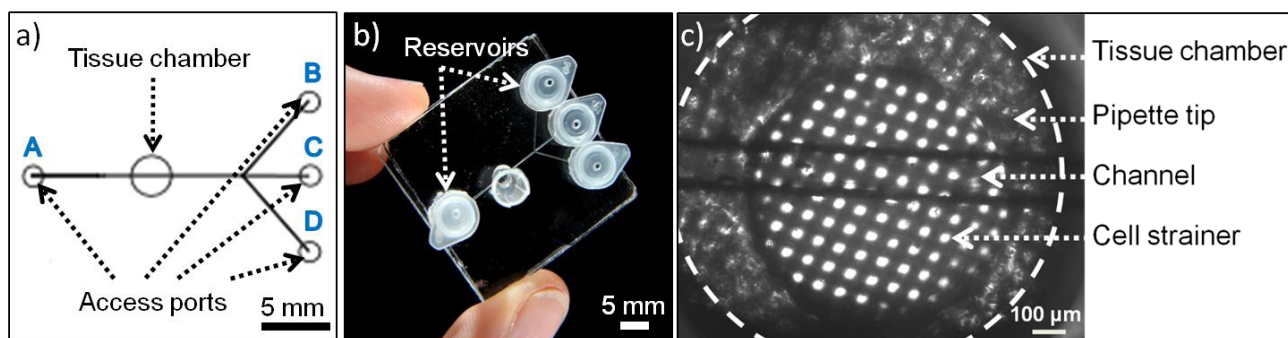


Figure 2: Microfluidic device. a) Scaled schematic showing channel layout, tissue chamber & access ports A – D. b) Photograph of device with reservoirs attached. c) Micrograph of $50 \mu m$ mesh cell strainer in tissue chamber.

Channels were filled with 0.25 % (w / v) low melting point (LMP) agarose gel [A9419 Sigma-Aldrich, UK], which was made using 0.025 M HEPES / 0.25 M sucrose. Anonymised tumour samples were obtained from patients undergoing surgery, having gained ethical approval from Hull and East Yorkshire Research Ethics Committee (07 / H1304) and the Hull and East Yorkshire Hospitals NHS Trust (RO568). Samples were stored in Dulbecco's modified Eagle's medium [DMEM; E15-009, PAA Laboratories Ltd, UK] at $4 ^\circ C$, and if not used immediately, snap frozen and cryopreserved in liquid nitrogen at $-196 ^\circ C$. Tumour tissue ($\sim 1 \text{ mm}^3$) was placed upon a $50 \mu m$ cell strainer [340632, BD Falcon, UK] secured within the tissue chamber (as shown in Figure 2c) and submerged in $200 \mu l$ DMEM containing collagenases together with antibody against integrin subunit $\beta 1$ (implicated in metastasis). The device was incubated at $4 ^\circ C$ for 24 h. Excess enzyme / antibody mixture was then replaced with buffer, and the device incubated at $37 ^\circ C$ for 30 min, before being placed upon the stage of an inverted fluorescence microscope [Zeiss Axiovert S-100]. Once cell motion had subsided, an electric field was applied *via* 0.5 mm Pt electrodes submerged in reservoirs A and B (Figure 2a & 2b) and gel-based electrokinesis employed to transport the dissociated labelled cells along the microchannel. Image sequences were captured by monochrome CCD camera [Hamamatsu Orca ER] and analysed in ImageJ to derive v_{app} and μ_{app} for tumour cells labelled and dissociated as above, and also (in separate experiments) for unlabelled K562 cells.

RESULTS AND DISCUSSION

Following removal of the perfusate, incubation at $37 ^\circ C$ increased enzyme activity *within* the tissue; ECM fibres were digested and labelled cells released, whereupon the flow of dissociated cells through the cell strainer was assisted by gravity and hydrostatic pressure. Concurrently, gel in the channel began to melt, permitting cells to enter the macropores. Antibody-labelling of cell membrane-expressed cancer-associated targets was thus achieved *in situ* during interaction with neighbouring cells and tumour-specific ECM, providing a more accurate reflection of *in vivo* tumour cell phenotypes compared with conventional protocols. Furthermore, Figure 3 shows that cells in the channel remain fluorescently-labelled and detectable above the background antibody during electrokinesis.

HEPES / sucrose gel facilitated minimal hydrodynamic back pressure during EOF, and also consistently generated anodic EOF conditions. Cell velocities comprised both dominant EOF bulk flow and opposing cell electrophoresis. Labelled fragments – with greater charge-to-size ratios – were observed moving *against* the bulk flow by dominant electrophoresis. For intact labelled cells, mean μ_{app} was $-0.8 \times 10^{-8} \text{ m}^2 (\text{V s})^{-1}$. In contrast, experiments using unlabelled cells resulted in mean $\mu_{app} > -2.0 \times 10^{-8} \text{ m}^2 (\text{V s})^{-1}$. Cells with bound conjugated antibody have increased cellular zeta potentials and greater charge to size ratios than unlabelled cells [6]. Labelled cells are consequently more electrophoretically mobile than unlabelled cells and, in anodic EOF, are impeded to a greater extent.

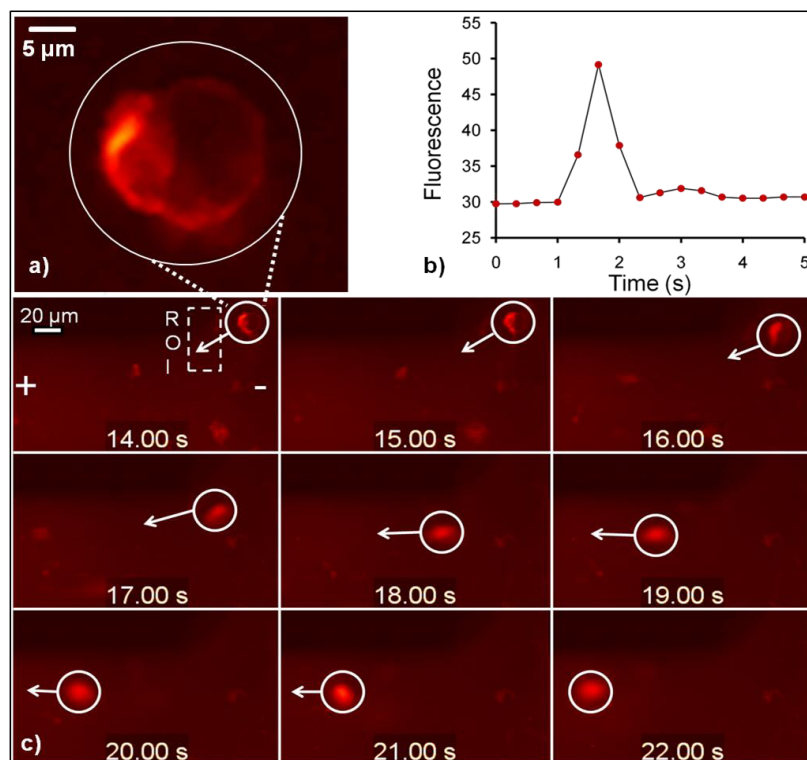


Figure 3: Electrokinesis of PerCPE-Fluor 710[®] anti- β 1-labelled and dissociated primary tumour cells in gel-filled microchannel (pseudocolour added): a) Fluorescence micrograph of stationary cell immediately prior to application of electric field. 45 V cm^{-1} was applied at 14s; b) Plot of intensity vs. time for cell traversing region of interest (ROI); c) Fluorescence micrograph sequence of cell electrokinesis. $\mu_{app} \approx -1.3 \times 10^{-8} \text{ m}^2 (\text{V s})^{-1}$

CONCLUSION

This innovative approach demonstrates for the first time the feasibility of using *ex vivo* tumour tissue comprising heterogeneous cell populations for microfluidics-based research into solid tumour biology at the cellular level. In addition, we have demonstrated the feasibility of integrating antibody-labelling with enzyme suffusion of tissue in preparation for rapid dissociation of labelled cells. The interplay between electroosmosis and electrophoresis for labelled tumour cells has been partially elucidated, and these findings inform the design and operation of a recently-built electrokinetic fluorescence-activated cell sorter (μ FACS), with potential for wide-ranging clinical applications, e.g. personalised diagnosis, prognosis and drug response monitoring.

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CONTACT

*J. Greenman, tel: +441482 466032; J.Greenman@hull.ac.uk