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Recent Developments in Cell-Based Microscale Technologies and Their Potential Application in Personalised Medicine

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1. Introduction

It is becoming increasingly apparent that some individuals are more susceptible to disease than others and more importantly some patients respond to prescribed therapies better than others. One of the main reasons for differences in disease susceptibility and the effectiveness of drug treatment lies in the genetic makeup of the patient. In addition to many environmental factors, genetic variations such as mutations, DNA polymorphisms and epigenetic gene regulation are the key players involved in the fate of a person's health. Recent advances in genomics and proteomics are providing novel insights into the complex biological process of disease. These insights will ultimately help to tailor personalised approaches to the treatment of disease based upon individual molecular "blueprints" of their genome and proteome.

Personalised medicine extends beyond the traditional medical approach in the treatment of patients as it aims to identify and target molecular factors contributing to the illness of individual patients. The personalised medicine approach is already playing a significant role in the way we treat and monitor disease. As many as 10 out of 36 anti-cancer drugs approved by the European Union in the last 10 years are considered to be personalised medicines (Eicheler, 2010). Breast cancer is one of the best examples whereby a personalised medical approach is adopted to detect the expression status of an oestrogen receptor called ESR1 in the nucleus of breast cancer cells. Approximately 70% of breast cancer patients overexpress this protein which is an important prognostic and predictive marker. Outcomes for these patients have been significantly improved by targeting the ESR1 using a hormonal treatment known as Tamoxifen. Interestingly this is the most commonly prescribed anti-cancer treatment in the world, highlighting the importance of a personalised approach in the management of disease.

Microscale technologies are emerging as an enabling platform for the development of novel personalised medicines and their broad accessibility. Miniaturised devices have the potential to process minute clinical samples and perform extensive genetic, molecular and cellular analyses directly on a microfluidic chip. The integration of pre-analytical sample handling with a subsequent sample analysis on a single microfluidic device will help to achieve highest reproducibility of results and minimise inter-laboratory bias and operators

errors. This will enable rapid investigation of drug effects on normal and diseased cells and help to assess the optimal dosage and the combinations of drugs to be prescribed for each individual patient. Furthermore, modern microfabrication processes enable mass-production of low-cost and disposable microfluidic devices making the new therapies more affordable.

In this chapter we discuss the emerging microscale technologies and their potential impact on the future of healthcare. We present the upcoming challenges and potential solutions for personalised medical technology which is currently being developed at the Biomedical Diagnostics Institute (BDI) in Dublin, Ireland. This chapter will focus on microfluidic assays for cell-based analyses and will demonstrate the efficacy of novel cell capturing techniques with particular emphasis on the detection of ESR1 in breast cancer cells.

2. Microscale technology

Since its origins in the 1980s, microfluidics has evolved into an exciting branch of biomedical engineering. The growing interest in microfluidics is largely due to its potential to revolutionise conventional laboratory handling, processing and bioanalytical techniques. A major advantage is their miniaturisation, enabling nano- and picolitre volumes to be processed. In the conventional laboratory setting micro- to millilitre volumes are routinely handled; however, by significantly reducing this volume, reagent consumption, assay time and the related costs are significantly reduced.

An important feature of microfluidic technology lies in the design of the microfluidic channels. Owing to their small dimensions, fluid flows in a strictly laminar i.e., essentially without turbulence. Mixing under laminar flow conditions is governed by mere diffusion of molecules across the phase interface (Hessel *et al*., 2005). The laminar character in microchannels can be harnessed for fluid control within, e.g. for fine adjustments of concentrations of molecules or cells over spatial and temporal microenvironments. As a consequence, new cellular applications are made possible with the unprecedented capability of closely mimicking *in vivo* conditions whereby cells are exposed to well-defined chemical gradients and changing microenvironments (Englert, 2009; Yu, 2005). These new and exciting capabilities become valuable to personalised medicine, both, from the point of view of basic research in cancer biology as well as for drug efficacy studies (Kang *et al*., 2008). The process of adaptation of cancer cells to altered microenvironments *in vivo*, in particular to hypoxic conditions, is still not fully understood. Microfluidics can provide a more in-depth insight into cell responses under these conditions mimicking specific microenvironments on chip (Polinkovsky *et al*., 2009). Microfluidic devices could therefore enable the study of combined effects of altered microenvironments and anticancer drugs on tumour cells and help to understand why anticancer drugs lose effectiveness in solid tumours over time (Minchinton & Tannock, 2006).

High level of parallelisation in microfluidic systems is another important feature which allows the investigation of a large number of experimental conditions at the same time, thereby reducing time and costs compared to conventional laboratory settings. The benefits of parallelisation in concert with the miniaturisation make microfluidic devices an excellent tool for high throughput analyses. This is a fundamental advantage for disciplines such as genomics and proteomics as they rely on large-scale analysis of genes and proteins. High throughput techniques provide also a sound foundation for personalised medical research, as large numbers of tests at various conditions are required when studying the effects of

drugs. Microscale devices, also known as Lab-on-a-Chip, can integrate several laboratory unit operations (LUOs) on just one miniaturised platform. The high degree of integration of independent LUOs using microfluidics has the potential to revolutionise personalised healthcare medicine through drug discovery (Dittrich & Manz, 2006) and point-of-care diagnostics (Yager *et al*., 2006).

Finally, but not of less importance, many novel fabrication methods are continuously being developed. Microfluidic devices are often made of polymers using mass production processes such as injection moulding and hot embossing which are optimised for microscale dimensions (Voldman *et al*., 1999). These microfabrication methods allow the devices to be produced in large volume and at low cost, which can potentially impact on global health, providing the opportunity to fabricate portable and disposable point-of-care devices for diagnostics applicable in poorly equipped environments.

3. Biomedical applications

Microscale technologies have significantly contributed to numerous biomedical applications over the past two decades. Encouraging advances brought by genomics and proteomics are helping to better understand complex molecular mechanisms of diseases. However, there is a growing need to translate results from genomic and proteomic research studies into clinical practice. This can be achieved by breaking barriers across disciplines and integrating various microscale technologies. Molecular profiling technologies are therefore adopting the microfluidic approach to solve challenges not amenable to conventional laboratory methods (Wlodkowic & Cooper, 2010).

The sequencing of the human genome has immensely increased our knowledge on human health and disease. Genome-wide analyses can now be performed with microfluidic devices for on-chip DNA amplification, electrophoresis and DNA hybridisation on microarrays (Yeo *et al*., 2011). Incorporating microfluidic technology not only improves conventional methods by reducing diffusion distances and assay times (Wang *et al*., 2003), but it may also significantly enhance assay sensitivities (Liu & Rauch, 2003). The most recent advances in microfluidics allow patient specific genetic analyses, such as whole-genome haplotyping from a single cell (Fan *et al*., 2011). Although many of the genomics platforms for the analysis of nucleic acids are still at research stages, some are particularly far advanced and ready for clinical application.

Microfluidics based proteomics is by far more challenging compared to on-chip genomics (Yeo *et al*., 2011). Proteins consist of polymers comprising 20 different L-α-amino acids and require a three dimensional globular structure to retain their function and activity. In addition, purified protein quantities are often limited due to the lack of simple methods for amplifying proteins similar to the powerful polymerase chain reaction (PCR) technique for nucleic acids. Despite the challenges with protein-based microfluidic devices, several applications for protein analysis have been developed including protein microarrays (Alvarez *et al*., 2008; Avseenko *et al*., 2002), chip-mass spectroscopy interfaces (Lazar *et al*., 2006) protein crystallization (Du *et al*., 2009) and most recently devices for monitoring of temporal expression events in immune cells within a clinical setting (Kotz *et al*., 2010).

The microfluidic approach to genomics and proteomics has the potential to help molecular profiling technologies to reach the maturity required for tests in clinical practice. It may pave the way towards the development of novel medical devices which utilise minute

quantities of patient sample to analyse DNA and protein signatures in high throughout systems. Furthermore, these novel bioassays may potentially allow preliminary self-screening or even basic treatment by front-line nursing staff, reducing the burden on practitioners and hospitals.

4. Novel approaches for cell trapping on a microfluidic chip

Microfluidic devices offer a unique opportunity to investigate individual cells derived from patients' samples. Subsets of cell populations involved in pathological processes can be monitored and a personalised medical approach can be tailored individually to the patient's needs. Although many different microfluidic cell trapping techniques are currently available, they frequently encounter problems such as low cell capture efficiencies, cell impairment through non-physiological shear stresses and limited measures of on-chip molecular analyses.

Immobilisation and contact–free cell trapping are the two main cell capture methods which are routinely used in microfluidics (Johann, 2006). Both techniques provide unique advantages with regard to the capturing of individual cells. Cell immobilisation utilises chemical and/or hydrodynamic approaches to trap cells efficiently. The chemical approach is based on antibody-protein interactions, whereby cells are immobilised onto surfaces which are micro-patterned with antibodies directed against specific proteins expressed on the surface of the cell (Anderson *et al*., 2004). The micro-patterning techniques provide high spatial resolution of immobilised cells and allow monitoring of individual cells in response to soluble stimuli. The hydrodynamic approach for immobilisation-based cell trapping relies on three dimensional surface topography microstructures to sieve cells from fluid flow in a microfluidic cavity. Mechanical barriers such as walls or micropores are utilised to retain the cells at rest next to a moving fluid (Khademhosseini *et al*., 2005). One of the main advantages of hydrodynamic trapping is its rapid cell immobilisation compared with chemical trapping methods as well as the often simple and inexpensive design.

In contrast to cell immobilisation, contact-free cell trapping uses magnetic, acoustic, dielectrophoretic and optical capture techniques to separate and handle cells (Johann, 2006). The contact-free techniques allow versatile and flexible cell handling, enabling cell positioning, holding, sorting and release with high accuracy and high selectivity (Werner *et al*., 2011). A possible disadvantage of the contact-free techniques is that cells are maintained in suspension which prevents adherent cells to grow in cell culture, thereby limiting contact-free trapping to bioanalytical applications. In addition, cells are exposed to certain electromagnetic or mechanical forces and to slightly increased temperatures which may have an undesirable effect on the analysed clinical specimen.

In the following section, we describe two novel hydrodynamic trapping methods which employ a sedimentation approach to capture micrometer-sized beads and cells. The first method allows the capture of beads within a microscale V-cup array based on a centrifugally driven sedimentation. The second method utilises gravitational sedimentation to capture cells within a microfluidic trench structure. Both methods facilitate particle capture with exceptionally high efficiencies and minimal exposure to hydrodynamic shear stress. We show on-chip molecular analysis of the breast cancer related oestrogen receptor ESR1 in cell lines as an example for potential personalised medicine applications.

4.1 Bead capture and analysis on a centrifugal microfluidic chip

Although various methods are available for actuating small volumes of liquids in microfluidic devices, the centrifugal microfluidics "lab-on-a-disc" approach offers a unique platform well suitable for high-performance point-of-care testing. In addition to forces present in most microfluidic systems such as capillarity, the actuation principle utilises rotationally induced inertial forces such as the centrifugal, Coriolis and Euler forces to move fluids and particles. Under the impact of the centrifugal force, fluids are propelled from the centre of rotation to the outer rim of the chip by an "artificial gravity" encountered in the rotating system. The centrifugal force scales with the square of the rotational frequency and is proportional to the distance from the centre of rotation as well as the radial length of the liquid plug. This allows controlling of flow velocities of liquids within the chip by using different rotational frequencies.

A major advantage of this approach is that it enables the design of systems consisting of an integrated drive unit, i.e., a motor with a self-contained disposable chip which is advantageous when dealing with clinical samples such as blood. Furthermore, the centrifugal pumping is widely independent of the physical properties of the liquids such as viscosity, conductivity, surface tension and pH. This feature is especially interesting for biological applications where samples with a broad range of viscosities and pH values need to be processed. Another unique feature of the centrifugal platform is that sample preparation steps such as separation of plasma from whole blood can be readily implemented by virtue of the density difference between cells and plasma. A comprehensive portfolio of LUOs such as valving, mixing and metering has already been demonstrated, as well as their integration into full-fledged sample-to-answer systems. For reviews of centrifugal microfluidic platforms see Ducrée, 2007 and Madou, 2006.

The particle trapping method presented here utilizes V-shaped retention elements often used in pressure driven microfluidic systems (Di Carlo *et al*., 2006). The centrifugal disc and the particle capture concept are shown in Fig. 1. Briefly, the V-cups are arranged in an array format such that there are no direct radial pathways between sample inlet and the end of the array. During the capturing process, the particles sediment through the array and are trapped when hitting a V-cup structure. Once a cup is occupied a particle, subsequently arriving particles deflect from the bulk and get trapped in subsequent cups. By scale matching the size of the V-cups to the size of the particles as well as the total number of particles introduced with the suspension, the occupancy distribution of particles per cup can be adjusted, even to a sharply peaked single-occupancy distribution.

A major improvement of the centrifugal V-shaped retention scheme is the absence of dynamic flow lines which are inherent to pressure driven systems. The dynamic flow lines within the liquid often drag cells suspended in the flow around the V-shaped structures, thus leading to low capture efficiencies of 20% and lower (Kim, 2011). In contrast, the centrifugal microfluidic device presented here sediments cells under stagnant flow conditions. Thus, suspended particles follow straight (radial) paths, with theoretical capture efficiencies of 100%. In experiments performed using 10-µm silica beads spun at a rotational frequency of 20 Hz, we obtained capture efficiencies greater than 95%. Although there are no dynamic flow lines under stagnant flow conditions, additional effects such as the surplus of particles captured in one V-shaped retention element, other impact factors such as the lateral Coriolis force may deflect the sedimenting particles, reducing the overall capture efficiency.

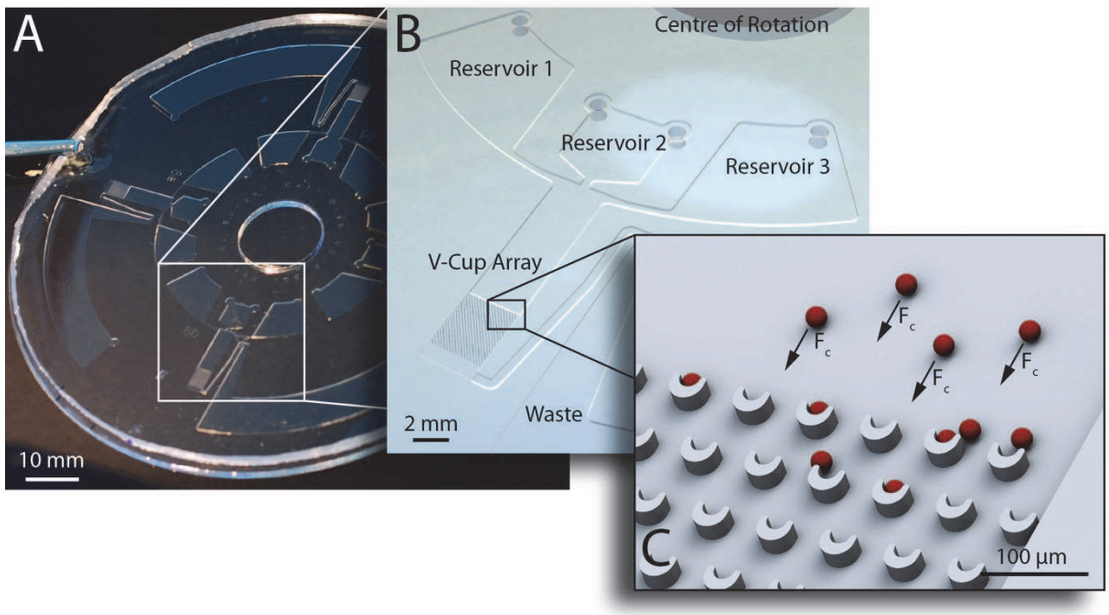

Fig. 1. Microfluidic V-cup array on a centrifugal platform. (A) Disc-shaped chip with four identical bead capture structures. (B) A drawing showing the design of one of four bead capture structures. (C) Magnified view of a V-cup array designed for sedimentation-based particle capture induced by centrifugal forces.

In addition to high capture efficiencies, this centrifugal device provides further benefits when compared with microfluidic bead-bed based immunoassays. In fact, beads introduced by flow towards a geometrical retention barrier tend to assume random aggregation patterns, which provide poorly defined, inhomogeneous flow and assay conditions for each bead. Moreover, in other multilayer arrangements, captured beads are located in individual focal planes making the readout more difficult. In contrast, using this novel device, the location of beads is given by the position of the capture structures, leading to precise flow control in the vicinity of each bead. Furthermore, all beads are located in the same focal plane which facilitates optical readout by a microscope. Experiments were carried out to demonstrate the importance of scale matching between capture element and particles. It has been demonstrated that the occupancy distribution of captured beads in V-cups peaks at single occupancy when the ratio of cup size to bead size is close to unity. We experimentally achieved a single particle occupancy of more than 95% of all occupied V-cups (Burger *et al.*, 2011).

The main feature of the centrifugal chip is the highly efficient capture of cells from clinical samples and subsequent molecular analysis on the chip. On-chip separation of cells allows discriminating between cell types and enables multiplexed immunoassays. In order to demonstrate its ability to separate and pinpoint particles to a specific location on the V-cup array, the device was loaded with a mixture of polystyrene beads coated with either human or mouse IgG antibodies (Fig 2). The mixture of both bead types was trapped in the V-cup array. Individual beads were visualised using Cy5 labelled anti-human IgG (red) and FITC labelled anti-mouse IgG secondary antibodies (green).

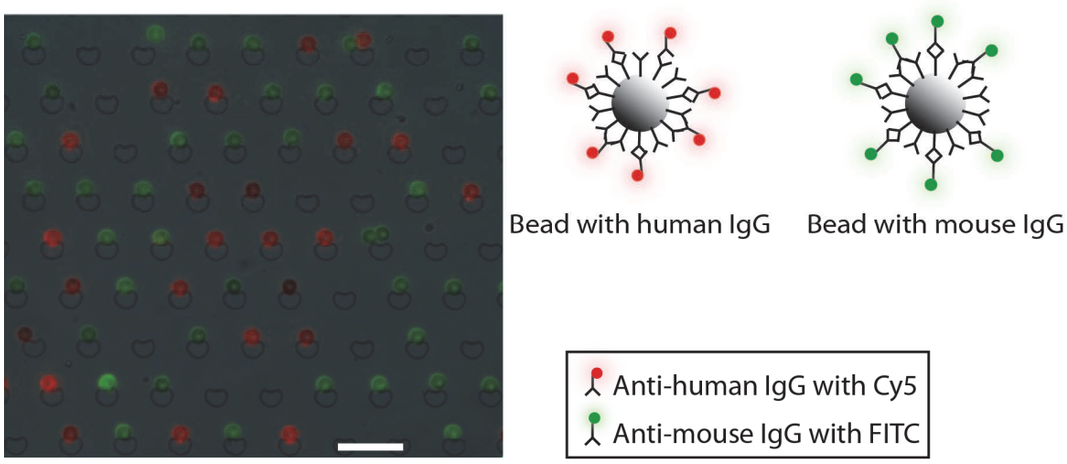

Fig. 2. On-chip immunoassay performed on the centrifugal platform. (A) Beads coated with human or mouse IgG antibodies were separated on the V-cup array and visualised using Cy5 labelled anti-human IgG and FITC labelled anti-mouse IgG secondary antibodies. The figure shows superimposed bright field, Cy5 fluorescent and FITC fluorescent images. Scale bar is 100 μm.

4.2 Cell capture and molecular analysis on a novel microfluidic trench chip

The second micro-particle capture approach which we recently developed utilises gravitational sedimentation in conjunction with a microfluidic trench structure for efficient cell capture and subsequent molecular analyses. The device was fabricated using standard soft lithography methods and consists of a network of microfluidic channels leading to a cell capture chamber. The design utilizes a microfluidic trench structure with characteristic dimensions (220 μm deep, 100 μm x 400 μm cross section) as a region of minimal flow for hydrodynamic cell capture (Fig. 3). Cells are loaded onto the microfluidic chip and dragged with the flow through the microfluidic channels into the capture chamber where the cells are effectively trapped. The widened section of the flow channel reduces the flow velocity, providing sufficient time for cells to irreversibly sediment into the trench. This is a highly efficient, merely sedimentation-based cell capture method, whereby experiments with HeLa and MCF7 cells show capture efficiencies close to 100% at flow velocities of 20 μm s^{-1} (Dimov *et al.*, 2011).

Cell loading onto the chip and flow velocities within the microfluidic channels are controlled by fluid levels within a pipette tip at the inlet of the chip. The pipette tip serves as an open liquid column generating hydrostatic pressure within the microfluidic channels. Flow velocities within the microfluidic channels and the trench structure were simulated using a computational fluid dynamics (CFD) approach (Fig. 3). The CFD simulation revealed decreasing flow velocities towards the base of the trench. Flow velocities at the bottom of the trench were calculated to be three orders of magnitude lower than in the channel above. Cells entering the low velocity region were therefore effectively retained at the base of the trench. Importantly, the minute flow velocities at the base of the trench significantly reduce shear stresses exerted on cells. Such shear-protected regions provide an advantage over other microfluidic cell retention methods, in particular in biomedical applications. Fluid shear stresses may considerably modify the state of captured cells and

introduce a bias into microfluidic bioassays. Minimising shear stress exposure may have a positive effect on microfluidic cell culture and opens up a route to analyse highly sensitive cells such as stem cells directly on this platform.

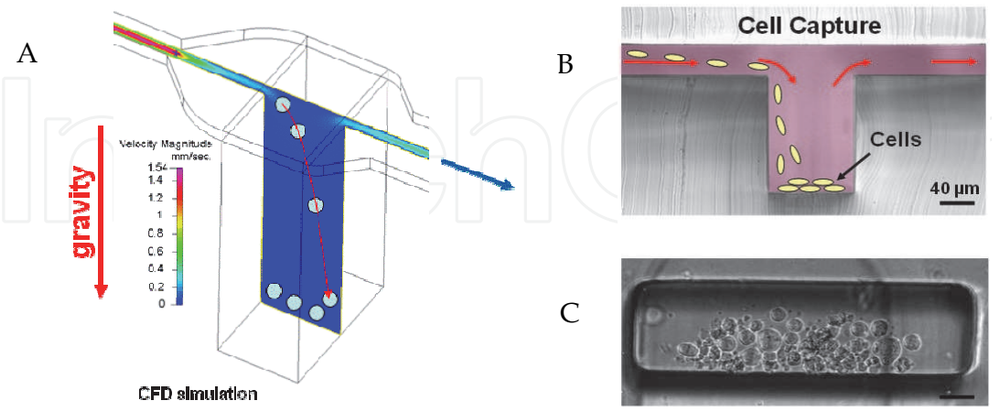

Fig. 3. Microfluidic trench structure: design and working principle. (A) CFD simulation of flow velocities within the trench structure. (B) Cells are captured based on the sedimentation of cells to the bottom of a microscale trench (side view). (C) Photograph of HeLa cells captured within the microfluidic trench structure (top view).

An important feature of the microfluidic trench device is its capability to perform several different bioassays in parallel (Kijanka *et al.*, 2009). Its key characteristic is a simple loading of liquids onto the chip, hence enabling rapid replacement of reagents within the trench for multi-step bioassays. Here we demonstrate an immunoassay performed directly on the chip. Cells and reagents were loaded onto the chip. The reagents were allowed to interact with captured cells through diffusive mixing within the trench structure. Finally, cell staining was visualised using a fluorescent microscope (Fig. 4).

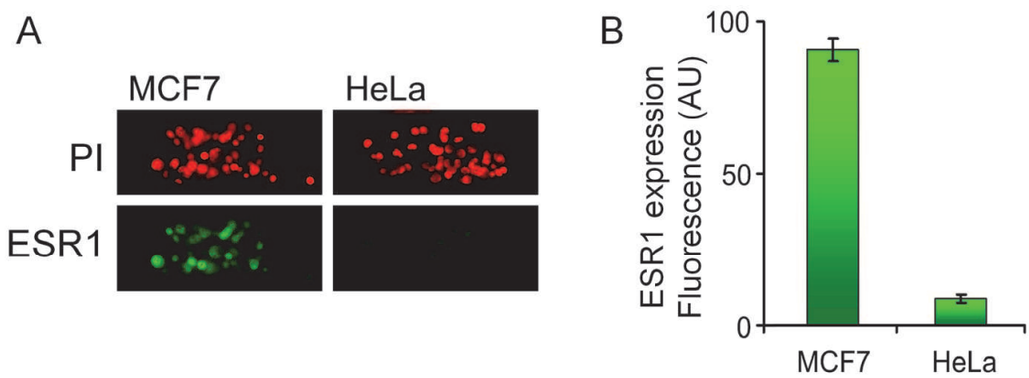

Fig. 4. On-chip immunoassay performed on the microfluidic trench platform. (A) MCF7 and HeLa cells were captured within the microfluidic trench structure. Cells were stained with propidium iodide (PI) to mark nuclei of all cells (red) and with anti-oestrogen receptor antibodies (ESR1) to visualise ESR1 expression (green). (B) MCF7 cells show specific nuclear staining for oestrogen receptor ESR1.

To determine the ESR1 levels in mammalian cells, cervical cancer cells (HeLa) and breast cancer cells (MCF7) were captured on the chip (Fig.4). Cells resting within the trench structure were exposed to a multi-step immunostaining protocol. Initially, cells were fixed with a 4% formaldehyde solution and permeabilised with ice cold acetone. These permeabilised cells were then treated with a 4% skimmed milk (Marvel) blocking solution to avoid non-specific binding. Both cells types were then incubated with a mouse anti-ESR1 antibody and corresponding anti-mouse secondary antibody labelled with the Alexa488 fluorophore. Since we expected a nuclear expression of ESR1, cells were counter-stained with propidium iodide (PI), a fluorophore with a specific red staining at cell nuclei. As shown in Fig. 4, both cell types were successfully immobilised in the microfluidic device and the immunostaining was performed. The counter-stain with PI revealed the location of nuclei within the cells (red). However, only the MCF7 cells, and not HeLa cells showed ESR1 expression when treated with specific, fluorescently labelled antibodies (green). The results show the ability of the device to perform complex molecular protocols directly on the chip. In this immunostaining experiment we could detect breast cancer related oestrogen receptor ESR1 in the breast cancer cell line MCF7 and the absence of this receptor in cervical cancer cell line HeLa.

5. Conclusion

Personalised medicine is gaining significant momentum in the medical field as a means to tailor patient care, based on a unique molecular signature. The application of novel methods to assess patient samples through minimally invasive technology will emerge as key tool in the diagnosis and monitoring of disease in the future. Low-cost, mass produced microfluidic devices have the capability to process patient samples in a highly efficient manner and enable the detection of markers of disease through the manipulation of cells under controlled microfluidic conditions. These technologies provide a suitable platform for the investigation of cells both on a genomic and proteomic level.

Current interdisciplinary research efforts focus on faster, accurate, reliable, and reproducible microfluidic tests applicable to clinical settings. In this chapter we described two novel approaches for cell capture and subsequent molecular analysis in a microfluidic chip. Both microfluidic devices demonstrate high particle capture efficiencies with the potential for application in diagnostic bead based immunoassays. Minimising shear stress exposure maintains the physiological integrity of cells within these microfluidic devices, thus helping to recreate *in vivo* conditions on chip. As personalised medicine emerges as the key approach to monitor and treat disease in the future, the accessibility and cost-effectiveness of these personalised tests will be critical for its success.

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