FISH and chips: chromosomal analysis on microfluidic platforms

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Abstract: Interphase fluorescence in situ hybridisation (FISH) is a sensitive diagnostic tool used for the detection of alterations in the genome on cell-by-cell basis. However, the cost-per-test and the technical complexity of current FISH protocols have slowed its widespread utilisation in clinical settings. For many cancers, the lack of a cost-effective and informative diagnostic method has compromised the quality of life for patients. We present the first demonstration of a microchip-based FISH protocol, coupled with a novel method to immobilise peripheral blood mononuclear cells inside microfluidic channels. These first on-chip implementations of FISH allow several chromosomal abnormalities associated with multiple myeloma to be detected with a ten-fold higher throughput and 1/10-th the reagent consumption of the traditional slide-based method. Moreover, the chip test is performed within hours whereas the conventional protocol required days. In addition, two on-chip methods to enhance the hybridisation aspects of FISH have been examined: mechanical and electrokinetic pumping. Similar agitation methods have led to significant improvements in hybridisation efficiency with DNA microarray work, but with this cell-based method the benefits were moderate. On-chip FISH technology holds promise for sophisticated and cost-effective screening of cancer patients at every clinic visit.

1 Introduction

Although human chromosomes have been studied for over a century, it was the introduction of fluorescence in situ hybridisation (FISH) analysis techniques, particularly interphase FISH, in the mid-1980s that allowed researchers to rapidly investigate and understand the chromosomal basis of many genetic diseases and cancers [1–4]. Interphase FISH is more sensitive than conventional cytogenetic methods for detecting chromosomal changes, such as the translocation (4; 14)(p16; q32) found in multiple myeloma (MM) patients. More specifically, probes separated by as little as 50–100 kb can be clearly distinguished as separate signals within interphase nuclei [3–4]. Since such changes are not easily detected by conventional cytogenetic methods, this technique has become an indispensable tool for gene mapping and characterisation of chromosome aberrations [5–7]. In addition, FISH provides resolution at the single cell level, enabling detection of rare event cells when sufficient numbers are screened. Interphase FISH also has a significant advantage for cancer testing because it avoids the need for proliferating cells to generate the metaphase spreads required by many cytogenetic methods. For many types of tumours, the inability to obtain chromosome spreads makes interphase FISH ideal for such cases. The technique can be applied directly to tumour samples (biopsies, sections and archived paraffin-embedded material) providing chromosomal information without the need for metaphase chromosome preparations [3].

In many cancers, the presence of known chromosomal abnormalities indicates the probable outcome of the disease and also predicts how patients will respond to therapy. The named translocation above, along with other abnormalities has been associated with lower survival rates, and patients harbouring these abnormalities do not respond well to conventional or high dose treatments [7–9]. Since some of the therapies have secondary effects that greatly compromise quality of life, it is necessary to determine the appropriate therapeutic approach for each patient. Consequently, FISH should be employed in a clinical setting to recognise, for instance t(4; 14)(p16.3; q32), allowing clinicians to make highly informed decisions regarding patient treatment. However, the complexity and numerous protocol steps involved in a typical interphase FISH analysis are labour intensive and time consuming, taking days to complete. Interphase FISH lends itself to automated approaches (such as dot counting) that reduce the analysis time required from highly skilled technologists [10]. A number of automated scanning systems are now available commercially, but visual confirmation of results remains important [3–4]. Even with automated image acquisition and classification systems, the cell preparation and probe hybridisation portions of the experiment take approximately 80% of the overall time. Furthermore, the probes required to perform FISH are relatively expensive (approximately $90 per slide). Together, these factors have prevented FISH from becoming a commonly employed screening technique in clinical settings. We demonstrate that the miniaturisation of FISH can reduce labour, time and cost to the extent that the more widespread application of microchip-based FISH can be expected in the future.

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The process of integrating and miniaturising conventional techniques onto microfluidic platforms is widely referred to as the creation of micro-total analysis systems (μTAS). It has been demonstrated that these are potentially superior platforms for biological assays when compared with many conventional analytical tools [11–12]. In μTAS, planar microchips incorporate a network of embedded microchannels that transport the sample from one manipulation to the next, enabling both precise control of reagents and automation of several consecutive steps [12–13], while leading to a significant reduction in total analysis time. For instance, hybridisation is the most time-intensive part of DNA microarray technologies and there are considerable research efforts aimed at improving the speed and efficiency of DNA hybridisation [14]. In traditional microarray hybridisation approaches, the reaction rate is limited by molecular diffusion; therefore, it takes a significant amount of time for the target to find and hybridise to its complementary probe [15–18]. To overcome this diffusion transport limitation, several groups have implemented electrokinetic or mechanical mixing of probes and targets on microchips [19–23]. The agitation introduced by these approaches results in a 2- to 20-fold reduction in hybridisation/analysis time.

When an electric field is applied during hybridisation, mobile DNA targets can be precisely controlled, thereby allowing continual replenishment or recirculation of targets to the immobile probes on the channel surface [19, 24–26]. In a recent example, Erickson *et al*. improved upon DNA microarray techniques by implementing an H-type channel fabricated on a glass and poly(dimethylsiloxane) (PDMS) microfluidic chip that permitted electrokinetic delivery of targets [19]. By restricting the channel height to 8 μm, they reduced the time it takes for a DNA target to vertically diffuse from the top of the channel to the bottom where the complementary probes are located and hybridisation can occur. When physical confinement is combined with a continual delivery of fresh targets by electrokinetic transport, the hybridisation time is reduced by 20-fold. Equally important advantages include smaller volumes of sample and reagent usage, portability, and high density parallel processing.

Alternatively, it has been demonstrated with DNA microarrays that volumetric flow can also be utilised to decrease the hybridisation reaction time. Kim [22] and Cheek [27] determined that a continual flow of targets at the highest volumetric flow rate and the lowest channel height yielded the fastest and most efficient hybridisation. Indeed, the concept is similar to electrokinetic pumping, employing a low channel height to minimise the vertical diffusion distance and a volumetric flow that provides a constant source of fresh DNA probes. Recently, mechanical pumps and valves have been incorporated within microfluidic chips, providing a high level of integrated fluid control [28–30]. One of the key benefits of these integrated and miniaturised valves and pumps is that they have lower dead volumes and therefore waste less of the expensive reagents.

Since conventional interphase FISH techniques depend on diffusion-limited hybridisation, there is potential for hybridisation enhancements. Although this may be true, in interphase FISH the samples are immobilised whole cells and chromosomes, as opposed to the short fragments used on DNA microarrays. Unlike DNA microarrays, the hybridisation process within a cell is substantially more complicated because the probes, which are on the order of kilobase pairs in length, must first diffuse to the cell wall, traverse it, and then find their specific binding site in a maze of 3 billion base pairs. With DNA microarray technology, it has been shown that as the number of hybridisation sites is increased (each site with a different sequence), the time taken for specific hybridisation increases significantly [31–32]. In the case of interphase FISH, by the same mechanism, the large range of distinct potential binding sites within a cell (orders of magnitude more dense than in DNA microarrays) may be expected to increase the time taken for hybridisation. Moreover, when targeting chromosomes with FISH, the hybridisation must occur within the physical volume of a cell nucleus and within packed-chromatin [4]. The diffusion is therefore hindered by the presence of RNA, enzymes, and various proteins, such as histones that bond to DNA. This cell to cell variability presents a challenging problem when modelling the mechanisms that determine the hybridisation rate. Clearly then, although interphase FISH is also dependent upon slow diffusion mechanisms, the process of hybridisation is far more complex than in DNA microarray work. Nevertheless, performing interphase FISH in the physical confinement of a microchannel permits the enhancement of the hybridisation kinetics and enables optimal reagent usage, leading to a reduction in cost and hybridisation time.

In this work, we describe the first microfluidic platforms to perform rapid interphase FISH analysis. Peripheral blood mononuclear cells (PBMC) were used for the detection of chromosomal abnormalities in malignant cells from patients with MM. Our initial design was developed to transfer the traditional FISH protocol into microfluidic channels with minimal changes to the method; the straight channel chip shown in Fig. 1*a*. This initial design has the same dimensions as the conventional microscope slide used in FISH, but it runs ten samples at a time with 1/10-th the reagent usage per sample. Once we had successfully developed and demonstrated a microchip-based FISH protocol, we were able to explore electrokinetic transport on the same microchip. We then investigated mechanical mixing with a newly created microfluidic chip that included pneumatic pumps and valves, shown in Fig.1*c*. Two microchip implementations were tested and both allowed us to reliably immobilise target cells, enzymatically treat them, controllably add DNA probes and enhance the hybridisation. This facilitated rapid FISH analysis. In either system, microchip-based FISH was completed in hours as opposed to the days required by the conventional approach and was more cost effective in terms of reagent consumption and labour.

1.1 Conventional interphase FISH

In a standard interphase FISH analysis [3–4], the cells under investigation are immobilised onto a glass microscope slide, often by cytospinning. Typically ~30, 000 cells are spun onto a microscope slide, of which ~6000 cells remain adhered after a FISH experiment, roughly 20%. For some protocols, the slides are then left at room temperature for a few days to 'age', which results in better hybridisation signals and stronger adhesion of cells [3]. Next, a proteinase digestion is performed to remove cytoplasmic and chromosomal proteins and RNA, improving accessibility to the chromosomal DNA. Following the digestion, the chromosomal DNA is dehydrated and fixed with a series of ethanol treatments that enhance the attachment of chromosomes and nuclei to the slides. The DNA probes are then added onto the slide and a coverslip is placed and sealed with rubber cement to prevent evaporation. Both the probe and chromosomal DNA are denatured (split into single stranded DNA) by heating the slide to a temperature of 75°C for 2–5 minutes. The slide is then

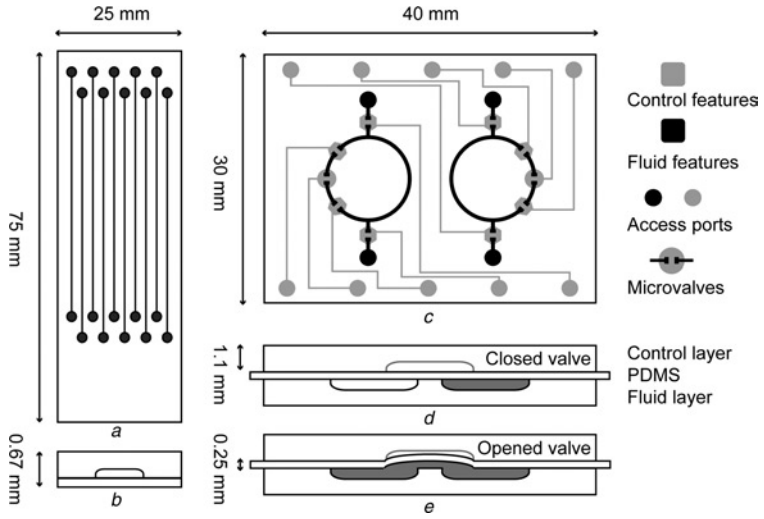

Fig. 1 *Two microfluidic chips used for interphase FISH analysis*

a Microchip array used to perform the microchip-based FISH protocol. This chip layout was also employed for the electrokinetic hybridisation enhancement protocol. The outer dimensions are identical to a microscope slide
b Sample crossection of a microchannel in the microchip array
c Combined mask layouts and dimensions of circulating microchip, which was employed for recirculating probes over immobilised cells. The grey lines are the features etched in the control layer for the pneumatic valves, whereas the black features are the fluidic network. The two glass substrates are sandwiched together with a thin PDMS membrane in between as shown; this creates a movable diaphragm
d Cross section of a valve in closed position as pressure is applied to the control layer on the top, thereby sealing the discontinuous fluid layer on the bottom
e Sample cross section of a valve in open position; vacuum applied on top side creating a fluid pathway on the bottom side

brought down to a temperature of 37°C and after a significant amount of time (typically overnight), hybridisation of probe DNA to the chromosomal DNA will be evident. To remove any cross-hybridisation (nonspecific binding), the slides are rinsed with a post-hybridisation solution. The cells are then analysed and classified (discussed below) by fluorescence imaging to yield a diagnosis [10]. Depending on where the probe hybridises along the chromosomal DNA, detection of various chromosomal abnormalities including deletions, insertions, and translocations is possible, giving interphase FISH techniques a broad range of capabilities for diagnostic testing.

One type of labelling strategy commonly used to detect translocations associated with MM is the 'Break Apart' probe (www.vysis.com). Conceptually, the chromosomal locus of interest is labelled with two different fluorophores flanking the spot where the break point is located. When a translocation occurs, one of the colours is left on the original chromosome, whereas the translocated portion with the other colour is found on another chromosome. Thus when imaging a cell, if the two coloured dots are close, there is no translocation, but when the coloured dots are far apart (greater than two signal diameters), a translocation exists.

2 Materials and methods

2.1 Reagents and solutions

Reagents were purchased from Invitrogen (Carlsbad, CA, USA) and all dilutions were performed with autoclaved Milli-Q water unless otherwise noted. Two buffers, 1 × PBS (phosphate buffered saline) and 2 × SSC (sodium citrate, sodium chloride, dilute from 20 × SSC stock), were used in these FISH experiments [3]. The enzyme solution employed for digestion was made by mixing 1 μL of 25 mg/mL proteinase K stock to 1 mL of 2 × SSC. The stock 95% ethanol was diluted to yield 70% and 85% ethanol solutions necessary for a series of dehydration and fixation steps; see procedure below. Five FISH probes from Vysis (Downers Grove, IL, USA) were used in our experiments to detect various chromosomal abnormalities associated with MM. Vysis does not disclose the concentration of their probes and all probes were used at the amount recommended by the manufacturer. Two of the FISH probes targeted for the immunoglobulin heavy chain (IGH) locus associated with 14q32 translocations were used; namely, the LSI®IGH/FGFR3 dual colour, dual fusion translocation Probe and the LSI®IGHC/IGHV dual colour, break apart probe. The three other probes are D13S319 to detect deletions of chromosome 13, and a mixture of CEP 17 and LSI p53 for chromosome 17. The probes were prepared as per the instructions provided by the vendor. NP-40, Nonidet P-40, a non-ionic detergent, was used in the post-hybridisation wash solution and for optimum probe performance. Vectashield H-1000 antifading solution was used to reduce photobleaching during fluorescence imaging and was purchased from Vector Labs Inc. (Burlingame, CA, USA). Ross rubber cement (Toronto, ON, Canada) was used to prevent evaporation during hybridisation by covering wells on a microchip and by sealing coverslips on a microscope slide.

The three cell lines used for experiments were RAJI (Burkitt's lymphoma), KMS12-BM (bone marrow, MM) and KMS18 (MM). Cell lines were cultured in RPMI 1640 + 10% fetal bovine serum (FBS) + 2 mM L-glutamine + 100 mM Hepes + 0.25 mg/mL gentamicine and maintained at 0.5–2million cells/mL; 5% CO_2 at 37°C. In addition, ex-vivo PBMC from three MM patients were purified as previously described [33–34] and used for microchip-based FISH.

2.2 Microchip array

The microchip-based FISH protocol discussed below was performed on a custom designed microfluidic device fabricated in the University of Alberta Nanofabrication Facility.

All microfluidic devices discussed were fabricated following standard glass fabrication protocols as described in [35–36]. The microfluidic network that implements the microchip-based FISH protocol is illustrated in Fig.1*a*. The microchip used here consists of 10 straight channels (nominal dimensions are 55 μm × 310 μm × 50 mm) and 20 wells (each containing ca. 1.5 μL). Channels are etched in 0.5 mm 0211 Corning glass (Precision Glass and Optics, Santa Ana, CA, USA) with access ports also in the 0.5 mm substrate. Although the 0.17 mm thick 0211 Corning glass cover plate requires careful handling during fabrication, it was necessary to create a minimum working distance for high resolution imaging.

2.3 Circulating microchip

This microchip was designed so that the probe could be recirculated over the immobilised cells, thereby facilitating more rapid and efficient hybridisation. This required a microfluidic network and a pneumatic pumping and valving system, as illustrated by the combined mask layouts shown in Fig. 1*c*. The concept is similar to the valves pioneered by the Mathies group [29] in that this circulating chip is built with three layers: a rigid bottom layer with fluid channels, a flexible middle layer that acts as a controllable membrane and a rigid top layer with control channels and chambers for actuating the valves and pumps. The bottom layer used here consists of two discontinuous circular fluid channels (nominal dimensions are 40 μm × 580 μm with a radius of 5 mm) and two wells (each containing ca. 1.5 μL) in 1.1 mm thick borofloat glass (Precision Glass and Optics, Santa Ana, CA). The middle layer was a 0.254 mm thin sheet of PDMS (HT-6135, Bisco Silicons, Elk Grove, IL, USA). The top layer was fabricated on 1.1 mm borofloat glass and had ten access ports drilled to provide individual control over each valving chamber. This allowed either pressure (15 psi) or vacuum (20 in.Hg) to be applied to a valve chamber, thereby closing or opening the valves, respectively.

These miniaturised valves are the key requirement for active mixing during the hybridisation phase of FISH. In traditional active mixing setups, a substantial volume of solution is contained off-chip, in the tubing and in the off-chip valves (effectively dead volume). When using relatively expensive reagents, such as the FISH probes, this conventional type of active mixing setup is uneconomical. Furthermore, it is difficult to maintain the off-chip solution at a uniform temperature. By integrating miniaturised valves on-chip we minimised the amount of expensive reagent used and we were able to uniformly control the temperature of the solution for denaturation and active mixing during the hybridisation process. Although several circulating channels can be chained in parallel with the same control lines for an increased level of automation, for research purposes we decided to implement individual control over each valve.

2.4 Equipment

The Microfluidic Tool Kit, referred to as the μTK, was purchased from Micralyne (Edmonton, AB, Canada) and provided electrophoretic control of reagents and DNA samples for the straight channel microchip array. The programmable application of high voltages to the microchip is fully controlled by the μTK via a compiled LabVIEW interface supplied by Micralyne [37]. A custom plexi-glass enclosure was built that mounted onto a thermocycler and provided voltages to the microchip while temperatures were being applied. Another custom desktop system was designed and implemented that permitted temperature control and automated pneumatic valve actuation for the circulating microchip. Additionally, we designed a software package to provide automated and programmable control of valves and temperature, permitting the pumping and valving to be repeatedly applied without human intervention.

Fluorescence microscopy was performed on a Carl Zeiss Axioplan 2 microscope (Oberkochen, Germany) with the appropriate filter sets. The objective used for imaging was a Carl Zeiss water immersion lens, the Achroplan 63 × W IR, with a numerical aperture of 0.9 and a working distance of 2.2 mm. This objective was necessary to resolve probes at a high magnification as it provided a sufficient working distance when imaging cells on the circulating microchip (relatively thick layer of glass 1.1 mm). Images were captured with Metamorph (v.7, Molecular devices, Downingtown, PA, USA.) and a Photometrics Cool Snap HQ charge-coupled device camera, 1392 × 1040 pixels (Roper Scientific, Trenton, NJ, USA).

2.5 Microchip procedures

2.5.1 Cell preparation: Before FISH could be implemented onto a microfluidic platform, it was necessary to develop a method for immobilising cells within channels. From an initial concentration of ~10 million cells/mL, ~15,000 cells suspended in 1 × PBS were added to each sample well (1.5 μL). Capillary force moved the cells (in solution) into the microchannel, coating the surfaces over entire length the channel. Any remaining cells in the wells were removed with a pipette. The microchips were then heated to an optimal temperature of 85°C for 10 min. The remaining cell solution in the channels was removed with a filtered vacuum line (20 in.Hg). After the temperature treatment, approximately 70% of the cells remained adhered to the channel walls, with almost 90% of the 70% on the bottom of the channel (etched surface). The temperature-induced immobilisation procedure was employed for all microchip experiments.

After immobilisation, the cells were enzymatically digested with proteinase K to allow the DNA probes to enter the cell. The proteinase K was delivered to the cells by pipetting 1.5 μL of solution into the sample well and allowing capillary forces to fill the entire channel. The proteinase K solution was left to digest cells for 10 min and then was removed with vacuum (20 in.Hg). The cells were then washed with a continuous flow of 30 μL of 1 × PBS through each channel to ensure enzyme removal. Next, dehydration and fixation of the chromosomal DNA were performed by a series of ethanol treatments. A 70%, 85% and then 95% ethanol solution was loaded into the channels and left for 1 or 2 min. Following the removal of the last ethanol treatment, vacuum was applied for 2 min to dry the cells. The following sections describe the three methods of hybridisation evaluated for the microchip-based FISH protocol.

2.5.2 Hybridisation with the microchip array: The probe solution indicated in the reagents section was then added to the sample wells of the chip and vacuum was applied to the opposite well to pull the viscous probe solution into the channel. The total volume of probe used was 1 μL, or 1/10-th that used on conventional microscope slides. All of the wells were then blocked with rubber cement to prevent evaporation. Next, a set of thermal sequences permited controlled denaturation of the

chromosomal and probe DNA. The program sequence was as follows: (a) 37°C for 5min; (b) 75°C for 5 min; (c) hold at 37°C. For diffusion-based experiments, the probe was left in the channel to hybridise for the time duration desired, which ranged from 1 to 14 hours. After the hybridisation time, 20 μL of 0.4 × SSC at 70°C was flushed through the channels. The channels were then emptied, and filled with 2 × SSC/0.01% NP-40. The solution was kept in the channel for 1 min. These post-hybridisation treatments ensured the removal of any cross hybridisation (non-specific binding). Next, the cells were washed with 30 μL of 1 × PBS in a continuous flow. Finally, the channel was filled with the anti-fading solution and imaging of the cells was completed with the fluorescence microscope indicated above.

2.5.3 Hybridisation with electrokinetic transport: Another set of microchip arrays, Fig. 1*a*, were also used for electrokinetic experiments and the chips were prepared as indicated above. However, after the thermocycling and before the hybridisation, the rubber cement was removed, hybridisation solution (provided by Vysis) was added to fill the wells, and the electrodes were lowered into the wells. An electric field sequence was applied to gradually move the DNA probes down the channel as follows: (a) the electric field (10 V/cm) was applied for 2 min in one direction; (b) 1 min pause; (c) the polarity was reversed and the field was applied for 1 min; (d) 1 min pause. The electric field program was continuously repeated in this manner for 1 and 4 hours. An electric field strength of 10 V/cm was chosen to ensure that the environment close to the cell was not significantly disturbed and to minimise the current draw. Following the electrokinetic cycling, the same post-hybridisation washes were applied to remove cross hybridisation and any remaining probe.

2.5.4 Hybridisation with a circulating microchip: The chip was prepared as mentioned above, with all the valves opened to allow the solutions to be passed through the channel. The probe solution was then added to the sample well of the chip and vacuum was applied to pull the viscous probe solution into the channel. With the channels now full, the two outer sealing valves were then closed to prevent evaporation. Next, the thermal sequence described above was applied. Once the temperature was set to 37°C, a pump-based hybridisation was performed. The peristaltic pumping was achieved by sequencing the three valves on the circular part of the microchannel in Fig. 1*c*. One pump cycle was completed every minute; see results section for the determination of pump frequency. The pumping program was repeated in this manner for 1 and 4 hours. After the hybridisation time, all valves were opened and the post-hybridisation washes and procedures were completed.

2.6 Image analysis

To validate the efficacy of microchip-based FISH, an objective method was needed to compare the amount of cell background fluorescence to the detected probe intensity in imaged cells. As such, a signal-to-noise ratio was algorithmically computed from microchip and slide based FISH images to measure hybridisation efficiency. We found that this signal-to-noise ratio was invariant to differing levels of image amplification, and proved to be an unbiased method to compare hybridisation across all physical platforms.

Each cell image was manually cropped for individual analysis from the full population image, with a buffer of at least 20 ambient background pixels between the observable cell edge and the image boundary. Cell images are typically comprised three regions (as discernible by eye). Ambient image pixels ($\boldsymbol{I}_a$) were defined as all contiguous pixels within 30 intensity levels (on an 8-bit greyscale) of an ambient reference pixel, that is the top-left corner of each cropped cell region. Pixels in regions of specific probe hybridisation ($\boldsymbol{I}_p$) were defined as all those contiguous pixels within ten intensity levels of a manually selected reference pixel within the region. The cell background intensity level ($\boldsymbol{I}_b$) was defined as the average intensity over all image pixels not identified as belonging to a probe or the ambient image background. Signal-to-noise values were computed as $\text{avg}(\boldsymbol{I}_p)/\text{avg}(\boldsymbol{I}_b)$ – the average intensity level of all probes within a cell (i.e the signal) divided by the background level for that cell (i.e the noise).

Although this simple process depends upon manual cropping and the selection of reference pixels, the use of all contiguous pixels minimises the effects of user selection. The remainder of the processing is entirely automatic. Future work will apply an automated image-processing method to identify cell/probe boundaries. Signal-to-noise evaluation was performed on 10–30 red and green channel sample images for each hybridisation condition (1, 2, 4 and 14 hours), and the average value of all samples (with standard deviations) at each hybridisation time was taken as the signal-to-noise ratio for that timepoint. Although this is a relatively small data set, the distribution appeared randomly distributed about the mean. This algorithm has been validated against human interpretation and provides an unbiased method of establishing the signal and noise levels.

When analysing FISH results by either method, a simple set of criteria was applied. Considering the regions of specific hybridisation to be circles, the circles had to be more than two diameters apart to be considered translocated. The cells having all of their red–green circle pairs closer than two signal diameters apart were classified as normal.

3 Results and Discussion

3.1 Immobilisation of cells

Frequently used conventional methods of immobilising cells for FISH include cytospinning onto a microscope slide surface, air drying of fixed cells deposited on slides or preparing tissue smears, but these types of approach are not feasible in sealed microchannels. Other techniques have been implemented for cell immobilisation including physical absorption, covalent and ionic binding (surface derivatisation), physical entrapment, dielectrophoresis and entrapment in gels [38]. Of these methods, physical absorption is the simplest technique to implement on-chip, but the immobilisation strength is comparatively weak. Initially, we attempted to use physical absorption by loading the channels with cells and allowing them to settle and immobilise. With a channel height of 20 μm, the cells clustered together and momentarily clogged the chip, preventing further reagent flow. However, they did not adhere and when vacuum was applied they were completely removed. Likewise, Gaver and Kute [39] performed a theoretical study on cell adhesion in a microchannel by varying cell size, channel height and flow rate. As the cell size became comparable to the channel height, adhesion rates dropped significantly [39].

The minimum channel height for adequate cell immobilisation while permitting reagent flow was in the range of 40–55 μm to implement physical absorption, but very few cells remained when the fluid phase was removed with vacuum. It is not uncommon for cytogenetics labs to 'age' cells with heat treatment. However, we discovered that heating the microchip also resulted in an increased number of strongly adhered cells. To investigate this effect further, we heated the chip to various temperatures and tested the three cell lines and cells from three ex-vivo patient samples at each temperature. The temperatures ranged from 55°C to 95°C, as very little adhesion occurred below 55°C and any temperature above 95°C was incompatible with later steps in the FISH protocol. Adhesion was assessed by adding the cells suspended in 1 × PBS to the channel by capillary force and counting the initial concentration. The temperature treatment was applied for 10 minutes and the chip was returned to room temperature at which point the remaining solution was removed by vacuum. The channels were then imaged to count the remaining cells. Fig. 2 shows the percentage of cells (from the initial concentration in the channel) that adhered to the channel walls at various temperatures. As the temperature increased, the average degree of immobilisation increased, and the standard deviation decreased. The standard deviations were calculated from three images of each channel over six channels (three cell lines and three patients) or $n = 18$; therefore the decrease in temperature signifies less variability in the percentage of immobilisation. The lower temperatures 55–65°C appeared to immobilise adequately, but when flows were introduced many of the cells dislodged, indicating only a slight improvement in adhesion. However, at 75–85°C, the cells were strongly adhered while maintaining the cell morphology. Above 95°C, we noted severe damage to the cell morphology to the extent that FISH analysis was not feasible.

The high level of immobilisation observed at 75°C (70%) significantly exceeds that for example of cytospinning (20%) and enables the detection of even fairly rare events, depending on the absolute number of immobilised cells within the channels. Within the physical space available on the bottom surface of the channel, we estimate that as many as 5000–10 000 cells can be immobilised, enabling detection of rare events. Use of multiple channels, and/or increasing the channel dimensions would increase the number of cells available for staining and scanning, thereby increasing the minimum frequency of rare events that can be detected on chip.

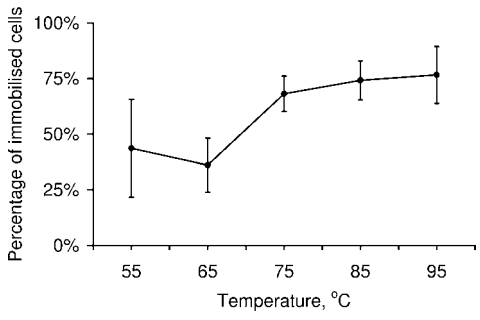

Fig. 2 *Percentage of cells immobilised on the bottom surface of the microchannel at various temperatures*

The above data set is the average of three cell lines and three patient samples (ex-vivo MM PBMC) with standard deviations ($n = 18$). Cells were added to the channel and the initial number was counted. Following the heat treatments, vacuum was applied and the remaining adherent cells were counted. Adhesion increases with temperature and the standard deviations decrease, allowing a more repeatable percentage of immobilisation. At 95°C the cells begin to burst, imposing an upper limit.

The fluorescence image in Fig. 3 illustrates a typical pattern of immobilisation. Our procedure, regardless of the sample, was able to reliably immobilise cells to cover at least 10% of the bottom surface area in a microchannel. Further, we found that almost 90% of the adhered cells (90% of 70%) were preferentially immobilised on the bottom surface of the microchannel (etched surface). The few cells that immobilised on the top surface or the side surfaces are visible in the fluorescence image (Fig. 3) by slight blurring. Cells immobilised on the side surfaces of the channels (noted by the apparent clustering) cannot be adequately assessed, and were excluded from the analysis. We also noted that cells adhered more densely to regions where the glass etch was rougher (coarse surface), such as the curved part of the microchannel (side surfaces). This phenomenon can be seen from the higher frequency of cells along each side of the channel in Fig. 3. We attribute this behaviour to surface energy minimisation along the curved portion of the channel. The adhesion to coarser areas was similarly observed when a section of the channel had a pattern of roughly etched glass, where the cells adhered more strongly and recreated the underlying pattern. This surface tailoring for site-specific immobilisation is presently being investigated in our laboratory.

The temperature immobilisation of cells eliminated the use of specialised equipment, namely the cassette cytospin centrifuge. Since heating also 'aged' the cells, better hybridisation and brighter signals were obtained without further treatment, such as waiting for a few days as is done in some conventional methods. The temperature immobilisation also preserves more of the three-dimensional cell structure, as discussed below.

3.2 Direct FISH implementation

Using a mixture of two cell types (normal and translocated), we verified that the temperature induced immobilisation did not significantly affect the FISH protocol. After hybridising the probe overnight, we imaged the cells within the microchannel on the fluorescence microscope and found that we had obtained comparable performance to the traditional method. As shown in Fig. 3, we were able to clearly distinguish normal cells from malignant cells. Furthermore, the microchip-based FISH used 1/10-th the probe, thus reducing the cost substantially as the probes are relatively expensive ($90/test down to $9/test). Subsequently, tests on a variety of cell lines and patient cells in combination with various probes to assess performance and ensure robustness of the protocol were performed. Table 1 summarises the combinations of cells and probes performed with microchip-based FISH, confirming the stability of the developed protocol with a variety of samples.

3.3 Hybridisation studies

Although the probes hybridised adequately, we noted that the microchip implementation had a slightly lower signal-to-noise ratio than the conventional microscope slide-based FISH. To study this difference further, we performed a series of time-based diffusion hybridisation experiments for 1, 2, 4, and 14 hours, as shown in Fig. 4. The 1- and 2-hour hybridisations for both microchip and slide methods yielded many cells that were hybridised; however, the degree of hybridisation was heterogeneous, with many cells having little signal. Although the 1- and 2-hours experiments provided valid detection of chromosomal patterns, the

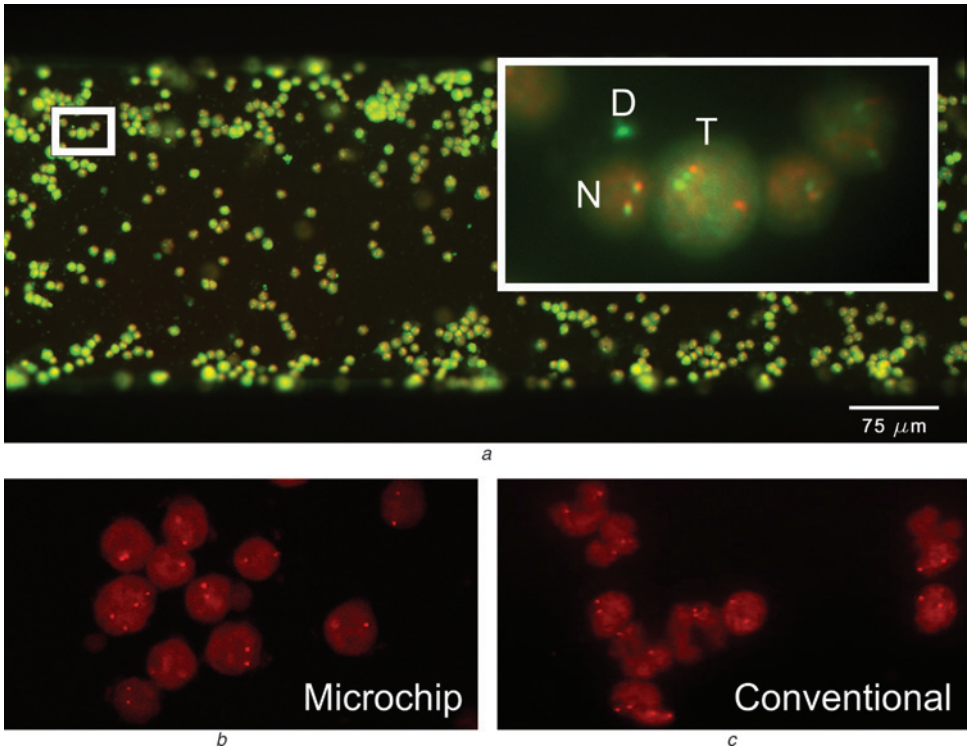

Fig. 3 *Fluorescence image of a typical pattern of immobilisation*

a Fluorescence image of microchannel after completing a FISH experiment on a microchip array with a hybridisation time of 14 hours (break apart probe). The cells imaged above are a mixture of KMS-12-BM (bigger cells that harbour a 14q32 translocation) and ex-vivo cells from a patient (smaller cells) with a normal chromosomal complement. The expanded image (white box) shows a cell (N) with two pairs of co-located red and green dots (indicating a normal chromosomal arrangement) and a cell (T) with one co-located pair of dots and a split pair with the red dot far from the other three (indicating a translocation). Debris (D) was found in both conventional and microchip images although more common in the latter. The remaining cells in the image are insufficiently resolved to analyse as the signals are in different focal planes, an occurrence common with both conventional and microchip FISH

b Fluorescence image of ex-vivo cells from a patient in a microchannel after completing on-chip FISH with a hybridisation time of 14 hours. With this single colour probe (LSI D13S319), a normal cell will have two red dots and a malignant cell will have only one red dot. The image has one malignant cell surrounded by several normal cells (one of the cells has four dots, possibly indicating that cell division was about to occur)

c Picture taken from conventional interphase FISH protocol completed on a patient sample with a microscope slide after 14 hours of hybridisation (image produced by commercial image acquisition system-BioView Duet scanning unit, Rehovot, Israel). The probe used was LSI D13S319 for detecting 13q14.3 deletions. Both *b* and *c* were representative images of their respective methods and either image was readily interpretable

4-hours experiments had a homogeneous degree of hybridisation. One trend shared throughout each hybridisation experiment was that the microchips had higher levels of background noise than the slides.

After imaging the microchip arrays and microscope slides on a confocal microscope, we determined that the cells retained more of their 3-D structure on the microchips, being less 'flattened' with the temperature immobilisation than with the cytospinning protocol used conventionally. Attachment of cells by cytospin centrifugation is known to cause substantial flattening of the nuclei [3]. The slight signal-to-noise variation in Fig. 4 may arise in part from the methods of immobilisation employed. The temperature-induced procedure preserved more of the intact cell structure, potentially creating higher levels of background fluorescence. Further, it becomes difficult to optimally focus on multiple probes to establish bright signals with a 3-D cell because there are more potential focal planes. With flattened cells most probe signals lay in the same focal plan with optimal intensity, but with 3-D cells the varying vertical positions of probes result in lower average signal intensity. Even with increased background

Table 1: Microchip-based interphase FISH with multiple cell and probe combinations

Cell	Probe[1]	Detected[2]
Patient 1	LSI IGH/FGFR3 dual color, dual fusion	t(4; 14)(p16.3; q32)
Patient 2	LSI IGH dual color, break apart	14q32 translocations
Patient 3	LSI D13S319	13q14.3 deletion
RAJI cell line	LSI IGH dual color, break apart	14q32 translocations
KMS12-BM cell line	LSI p53 and CEP17 D17Z1	17p13.1 deletion
KMS18 cell line	LSI D13S319	13q14.3 deletion
80% KMS12-BM + 20 % RAJI	LSI p53 and CEP17 D17Z1	17p13.1 deletion
80% Patient 1 + 20% KMS18	LSI IGH/FGFR3 dual color, dual fusion	t(4; 14)(p16.3; q32)

[1]Refer to reagents section for information on probes.
[2]Chromosomal abnormalities associated with multiple myeloma

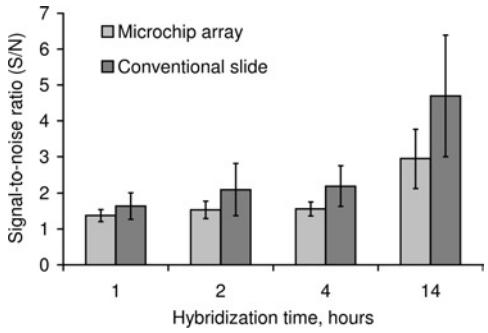

Fig. 4 *Signal-to-noise ratio (hybridisation efficiency) against the hybridisation time with a constant probe concentration; using the RAJI cell line and the break apart probe*

Microchip array offers comparable performance to the traditional method using 1/10th the reagents. The means and standard deviations were calculated as discussed in the image analysis methods section.

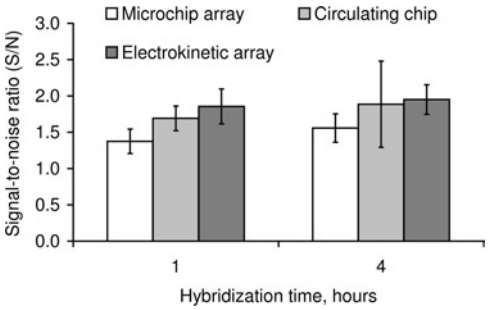

Fig. 5 *Signal-to-noise ratio (hybridisation efficiency) against the hybridisation time with a constant probe concentration; using the RAJI cell line and the break apart probe*

Mechanical pumping and electrokinetic transport of probes within the microchannel provide a ~30% improvement in the signal-to-noise ratio. The means and standard deviations were calculated as discussed in the image analysis methods section

and the 3-D cell structure, the probe signals were clearly distinguishable ($S/N \simeq 3$) permitting the images to be readily analysed by a FISH technician. The images were comparable in quality to images from conventional FISH, whether they were assessed by trained technician or the tolerance-based computer method we developed (see experimental section on image analysis). With the microchip-based FISH protocol established, we investigated electrokinetic and mechanical pumping of the probes to increase the hybridisation speed.

3.4 Hybridisation enhancements

Typically, DNA microarray technologies attain order of magnitude decreases in the hybridisation time when the solution is agitated [40]; however, other barriers to hybridisation within a largely intact cell present a substantially more complicated situation [3–4]. Although interactions with intracellular components slow hybridisation inside the cell, microchannels minimise and control the diffusion distance for probes outside the cell. The diffusion distance is minimised by confining the width and height of the microchannel, which are tunable to accommodate the procedures being performed, while cycling the probe solution along the channel. Pneumatic or electrokinetic pumping provides a good strategy for accelerating hybridisation, with a pumping cycle to ensure a significant concentration of probes local to the cells, followed by a 1min pause allowing enough time for probes to diffuse into the cells and hybridise. We estimated that the optimal pause time would be the time required for a probe to diffuse around a typical cell (circumference of 30–35 microns) along the surface of the channel wall using Einstein's diffusion equation [23]. The DNA diffusion coefficient used in our calculation, $1.0 \times 10^{-7}\,\mathrm{cm}^2/\mathrm{s}$, was consistent with commonly used values in typical DNA microarray analysis [20, 21, 41, 42] for similar strand lengths. As illustrated by the graph in Fig. 5, these agitation methods gave ~30% improvements in the signal-to-noise ratio. This corresponds to a four-fold decrease in hybridisation time, as the signal-to-noise ratios at 1 hour with pumping are equivalent or better than the diffusion-based hybridisation at 4 hours. These preliminary results are promising and the theoretical aspects of the hybridisation mechanisms [32] are under investigation. Characterisation of the mechanisms will permit optimised circulation schemes to enhance the hybridisation rate even further.

It should be noted that the homogeneity of probe hybridisation when employing mechanical pumping or electrokinetic transport was similar to diffusion-based experiments in that the 1-hour hybridisations were heterogeneous and the 4-hour hybridisations were homogeneous. We attribute this to variabilities in cell membrane permeability, resulting from the proteinase K treatment. This observation has also been documented with conventional FISH, in which the proteinase digestion has been the most difficult step to control as each cell is susceptible to the enzyme in different ways [4]. In the electrokinetic experiments, the channel walls near the electrodes maintained a high level of background fluorescence and cells closer to the electrodes appeared to have localised areas of non-specifically bound probes. Although the probe is not fully utilised in these regions, the chip and instrumentation will allow considerable flexibility in developing improved methods to optimise probe usage. We have also achieved similar results with probe dilutions of as little as 1/60, albeit with lower hybridisation speeds. These are promising levels of improvement in these initial tests. Clearly, a comprehensive survey of microchip-based cell preparation methods and hybridisation strategies is warranted. Currently, we are developing our own FISH probes with known concentrations that will permit us to quantify and further study the increase in hybridisation efficiency noted with microchip FISH. In addition, future work will involve strategies to further minimise the concentration/amount of probe required, for instance, by utilising arrays of physical cell traps in combination with probe recirculation to optimise delivery of probes to cells [43]. Such a survey is beyond the scope of the present report.

4 Conclusion

We have successfully performed FISH on a microfluidic platform for the first time. A novel method of immobilising cells to the microchannel surface was introduced and evaluated. We were able to detect a variety of chromosomal abnormalities associated with multiple myeloma with comparable performance to the conventional microscope slide-based FISH. Further, we have shown that there was a moderate enhancement in speed with mechanical pumping or electrokinetic transport of probes. The underlying mechanisms that limit the hybridisation rate are currently under investigation. These first on-chip demonstrations of FISH were completed in hours as opposed to the days required for the traditional method, while using 1/10-th the amount of reagent. The resulting cost reductions, and the many fold increase in throughput, are likely to lead to microchip-

based implementations of FISH in clinical settings where it would not otherwise be possible.

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