MICROFLUIDIC MODULE FOR BLOOD CELL SEPARATION FOR GENE EXPRESSION RADIOBIOLOGICAL ASSAYS

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Advances in molecular techniques have improved discovery of biomarkers associated with radiation exposure. Gene expression techniques have been demonstrated as effective tools for biodosimetry, and different assay platforms with different chemistries are now available. One of the main challenges is to integrate the sample preparation processing of these assays into microfluidic platforms to be fully automated for point-of-care medical countermeasures in the case of a radiological event. Most of these assays follow the same workflow processing that comprises first the collection of blood samples followed by cellular and molecular sample preparation. The sample preparation is based on the specific reagents of the assay system and depends also on the different subsets of cells population and the type of biomarkers of interest. In this article, the authors present a module for isolation of white blood cells from peripheral blood as a prerequisite for automation of gene expression assays on a microfluidic cartridge. For each sample condition, the gene expression platform can be adapted to suit the requirements of the selected assay chemistry.

INTRODUCTION

In the spectrum of a disaster response to a radiological incident, a rapid and accurate triage system for sorting of victims would be a necessity. In order to do so, a fully automated point-of-care system that could integrate an assay platform for processing a dosimetric assay would be highly desirable. The biomarkers linked to radiation exposure have now been well documented and are being used across the different platforms⁽¹⁾. The authors' team is developing the devices, biomarkers and bioassays for the accurate determination of an individual's absorbed dose of ionising radiation between 0.5 and 10 Gy. The most recent techniques to emerge are based on gene expression analysis. Changes in gene expression in the response of peripheral blood lymphocytes to radiation can reveal both whether an individual has been exposed to radiation and the magnitude of the dose received^(2–8).

This report describes a blood cell separation module for complete automation of sample processing for a quantitative nuclease protection assay (qNPA) microfluidic cartridge reported previously for performing gene expression-based biodosimetry⁽⁴⁾. The qNPA requires the separation of red blood cells (RBC) from white blood cells (WBC), which can be accomplished on a large laboratory flow cytometer or directly onto a cell separation micro-device. The purified leukocytes can then be used to follow gene expression changes via the qNPA chemoluminescent detection for correlating ionising radiation dose exposure for biodosimetry point-of-care measurements. Other assay chemistries can work directly on whole blood on bench top but

might still require prior removal of RBC to adapt the assay on a microfluidic platform.

The blood cell separation module uses a low-cost microfluidic plastic chip based on 'deterministic lateral displacement' (DLD) hydrodynamics, where cells with different sizes flow through a micro-post array with columns of the array tilted slightly to the cell stream and those larger than a 'critical size' defined by the array are bumped/displaced out of (and separated from) the original stream^(9, 10). No complicated equipment or labelling is required. Enrichment of WBC to RBC ratios of 110-fold and ~25 000-fold has been reported^(11, 12). However, all the previous DLD devices were made by deep reactive ion etching of Si wafer, which is an expensive process. Plastic chip should lower the cost dramatically but has not been demonstrated. Here, a DLD plastic chip is reported for the first time using photo-patterned SU-8 material to form an array of deep micro-posts between two cyclic olefin copolymer (COC) substrates for WBC separation from blood.

RESULTS

qNPA on whole blood

These experiments were approved by the institutional review board (IRB 2008–036) and were conducted according to the principles expressed in the Declaration of Helsinki. Written consent for participation in the study was obtained from all the subjects voluntarily.

It has been previously demonstrated that the bench-top qNPA can be performed on RNA or leukocytes purified from *ex vivo*-irradiated whole blood and that the qNPA can discriminate between non-irradiated and irradiated blood samples^(4, 5). In order to facilitate the automation of the assay, it was tested

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if the qNPA could perform as well directly on whole blood without the need of RNA extraction or leukocytes purification (Figure 1). After blood collection, blood was mixed directly with the 2X qNPA lysis buffer (1:1) using 15 μ l of blood for each sample.

The assay was run in triplicate for each sample. A very low gene expression signal response was observed for all the genes in the panel compared with the signals typically obtained from extracted RNA and/or leukocyte samples^(4, 5). This is likely resulting from the interference of blood components with buffers

and other reagents of the assay chemistry kit. A weak signal was also observed for the negative control (ANT) indicating a failure of the S1 nuclease reaction during the qNPA process. The S1 nuclease reaction degrades the single-stranded molecules (non-hybridised); when this reaction fails, a signal for the negative control will be observed as it is done here. In addition, a high standard deviation among the replicates for the same samples was observed, which would make it difficult to analyse the data reproducibly, limiting interrogating dose response for biodosimetry. These results showed that the qNPA requires preprocessing of the whole blood samples. In order to overcome this issue and in the view of automating the qNPA platform, a cell-sorting system was designed and built to separate the WBC from the RBC. This module will have the capability to be integrated into a microfluidic workflow processing cartridge readily available for interfacing with a rapid analysis system.

Figure 1. qNPA on whole human blood. Whole blood samples were collected from three volunteers (Vol 1–3). Transcript level of 15 genes normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured with qNPA run directly on whole human blood samples. The error bars represent the standard deviation between the three replicates run for each blood sample.

Leukocyte-sorting sample preparation module

Cell-sorting device

The leukocyte-sorting module is based on ‘deterministic hydrodynamics’ to separate blood cells by size. The design of the module is tuned to optimise the device stability and separation yield. The design has 16 sample injection channels, and each channel is sheathed by buffer to maintain a straight sample injection even under buffer/sample flow rate variation (Figures 2a and 3a). Figure 2b shows the image of a running leukocyte-sorting device on a fluorescent microscope

Figure 2. (a) Schematics of leukocyte-sorting device design and (b) image of a running leukocyte-sorting device laying horizontally on a fluorescent microscope stage.

Figure 3. (a) Bright-field images of sample injection and collection, (b) fluorescent image showing bumping trajectory of a white cell and (c) fluorescent image of white cells in collection channels.

stage. The device was horizontally oriented, and the sample and buffer flowed from left to right. The cell-sorting pillar array was designed to have a critical size of $\sim 4 \mu\text{m}$, with $18 \mu\text{m}$ pillar size, $17 \mu\text{m}$ gap between pillars and a row shift of $1/42$ of the array period.

SU-8 photolithography process was used to fabricate the device. After cleaning of the bottom COC substrate, SU-8 film was coated on the surface by spin coating. Photolithography was used to pattern the SU-8 film to form channels and sorting pillar arrays. Then, the top COC substrate with appropriate fluid via holes was bonded to the SU-8 layer by thermal bonding at $\sim 125^\circ\text{C}$.

For this design, the interface of the microfluidic channels to outside world is challenging. First, an array of buffer inlet holes and an array of waste outlet holes need to be formed on the top COC plate. computer numerical control (CNC) machining was chosen to drill the hole arrays since CNC is readily available to rapid prototyping. To minimise the footprint of the device and also consider the feature resolution of CNC machining, a centre-to-centre separation of 1.4 mm was used for the via-hole arrays with a hole diameter of $\sim 760 \mu\text{m}$. Different drilling and thermal annealing conditions were tested to minimise cracks of COC plastic between the adjacent holes. Before thermal bonding of COC/SU-8, the via-hole arrays were aligned to the patterned SU-8 structures using a mask aligner. The assembly was then transferred to a PHI-Tulip (City of Industry, CA) hot press for thermal bonding.

The via-hole arrays were connected to fluidic tubings through rectangular CNC machined plastic reservoirs. The reservoirs with barb fitting adaptor were UV glued to the chip. The two cell collection reservoirs were made by cutting the bottom of $300\text{-}\mu\text{l}$ centrifugation tubes, then gluing them to the chip. This way, vortex can be used to suspend the sorted cells in the reservoir for collection. The collection efficiency of this approach was characterised to be over 98% .

Sample preparation

For each run, $90 \mu\text{l}$ of finger-stick blood was drawn from consenting healthy volunteers, mixed with $10 \mu\text{l}$

of sodium citrate anticoagulant then diluted to $300 \mu\text{l}$ with Dulbecco's phosphate buffered saline. To image the cell behaviours inside the device and characterise the sorting efficiency, a protocol was developed to fluorescently label the double-strand DNA of nucleated blood cells using Hoechst 33342 dye. Blood/cell samples were stained at a dye concentration of $5 \mu\text{g ml}^{-1}$ at 37°C for 30 min. Either the total blood samples were stained to image the leukocyte movement inside the device or $20 \mu\text{l}$ aliquot was taken from blood sample/collected leukocyte, and then stained for WBC counting. The white cells were counted by a haemocytometer using a 4',6-diamidino-2-phenylindole fluorescent filter set. Figure 3a shows bright-field images of sample injection and collection regions of one channel. Only large white cells were bumped into the collecting channels. Figure 3b shows a fluorescent image of a white cell inside the device. Due to the fast moving speed, the trajectory of the cell was captured. Figure 3c is a fluorescent image of the white cells captured within the collecting channels on their way to the collection reservoirs. By counting the initial WBC number, collected WBC number, collected RBC number and assuming initial RBC of $5 \times 10^6 \mu\text{l}^{-1}$, the initial WBC collection efficiency was over 50% , and the reduction of the RBCs was $100\text{--}1000\times$. The WBC collection efficiency is lower than the $\sim 99\%$ efficiency previously reported by Si devices. The reduced efficiency may come from potential dimension change of the plastic channel during thermal bonding. A low-temperature bonding process may address this issue, and further optimisation is needed to reach the near 100% WBC collection reported by Si devices.

Gene expression profile from purified leukocytes

The cell pellets collected from individual cell-sorting devices were resuspended in $1\times$ lysis buffer. The qNPA was run using 200 000 cells purified from the device from a single experiment (Figure 4).

A specific gene expression profile was obtained with the qNPA with no presence of S1 failure (no signal for negative control ANT) in comparison with

Figure 4. Gene expression assays on WBC from cell-sorting devices. Three whole blood samples were passed through three independent devices. The purified cells were run using either the qNPA (single measurement from a single device) or the CLPA assay (two measurements from two devices). Five transcripts common to both assays (plus GAPDH) were measured. The error bar shows the standard deviation between the two CLPA experiments on cells collected from two independent devices.

the qNPA on whole blood (Figure 1). To demonstrate the validity of the cell-sorting device with multiple assays, the purified cells were also processed using a different assay chemistry, the Chemical Ligation Dependent Probe Amplification (CLPA) (RED-Dx kit DxTerity Diagnostics, CA) (http://dxterity.com/dx_direct.php)⁽¹³⁾ (Figure 4). A strong signal was obtained with the CLPA with no signal for the negative control. In addition, this gene expression profile is identical to the profile obtained when the CLPA is run on whole blood⁽¹⁴⁾. These results demonstrate that WBC can be purified from whole blood using the cell-sorting device and that these purified cells can be used directly for analysis using gene expression assays.

CONCLUSION

Today, multiple gene expression assays are available, but none of them has yet been adapted for fully automated biodosimetry. As an example, the CLPA is a commercially available assay chemistry test to measure the expression of RNA biomarkers directly from blood. This assay has been developed for bench-top use, but it will need to be fully adapted on cartridge in order to be used as a field-deployable platform in the case of a radiological or nuclear event. Similar microfluidic modules are currently being designed for the requirements of such an assay (data not shown).

This article presents a sample preparation module for the isolation of WBC from whole blood. The device uses a very small amount of blood that allows for minimally invasive blood collection via a finger stick. Typically, a RBC reduction by a factor of 100–1000× can be obtained. Although it is not possible to eliminate completely the presence of RBC, the small residual amount of RBC does not interfere with the

major chemical reagents of the assays. Currently, the WBC collection efficiency is not as high as the reported value by Si device. Further improvement of device fabrication and optimisation are needed for near-complete collection of WBCs. Nonetheless, even with the reduced WBC collection efficiency, repeatable gene expression pattern profiles were achieved using fabricated plastic chips. The reduction of the RBC by the plastic chips is comparable with those reported by Si devices.

This micro-device can purify the WBC, which can then be used directly as a biodosimetry sample to identify non-irradiated versus irradiated whole human blood samples. This device has the potential to be used for other biological assays that also require the purification of WBC.

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