A Micromachined Sparse-Cell Isolation Device: Application in Prenatal Diagnostics

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ABSTRACT

Fetal cells can be isolated using several enrichment methods, however, since no technique to specifically isolate and use them for genetic diagnosis has reached clinical practice, prenatal diagnosis currently requires invasive procedures. A micromachined device to fractionate whole blood using physical means to enrich for and/or isolate rare cell types from peripheral circulation was designed. It has arrays of four successively narrower channels, each consisting of a 2-D array of columns. Current devices have channels ranging in width from 15μm-2.5μm, and are 5μm deep. Fetal nucleated red blood cells were isolated from cord blood reproducibly and molecular analysis confirmed their origin without contamination from maternal DNA. Future studies will provide methods to detect fetal cells from maternal circulation using a non-invasive approach, the risk for fetal loss will be eliminated, and test costs will be decreased.

Keywords: prenatal diagnosis, fetal cell, biochip.

1 BACKGROUND

Prenatal testing is performed to detect genetic abnormalities in the fetus at various times during gestation (10-19 weeks). Currently, pregnant women are screened prenatally for the levels of biochemical analytes in the mother's blood and fetal anatomical features are assessed by ultrasound. These markers are used with maternal age, weight, ethnicity, and gestational age to predict the risk for fetal defects.

Further work-up that includes chromosomal analysis is only offered to women aged 35 years or older, to those with elevated serum levels of α-fetoprotein (AFP), to those known to carry a genetic disease, or to those who have suffered multiple miscarriages [1]. Such criteria thus exclude the majority of pregnant women. Today, 80% of babies born in the US with chromosomal abnormalities have younger mothers who are not eligible for routine prenatal diagnosis because of cost, reimbursement issues, and inherent risk to the fetus [2]. To complete clinical genetic testing, amniocentesis and chorionic villus sampling (CVS) are two methods used to sample fetal tissue.

In amniocentesis, a sample of the amniotic fluid is removed transabdominally under ultrasound guidance. Amniotic fluid is tested to more accurately determine the concentration of AFP. Elevated AFP levels are indicative of open neural tube defects and kidney disease. Mid-trimester amniocentesis adds 0.5 to 1% risk of miscarriage induced by leakage of amniotic fluid, infection, or fetal injury over the baseline 2-3% miscarriage risk of any pregnancy at this stage [3]. CVS involves biopsy of placental tissue and can be done transcervically or transabdominally. CVS also adds a 1% risk of fetal loss to the baseline miscarriage risk of 2-5% at 7-12 weeks of gestation [3]. CVS can result in misdiagnosis, (more so than amniocentesis), due to contamination of the placental tissue with maternal cells.

It is obvious that a non-invasive alternative to current procedures would represent a significant clinical advance. Fetal nucleated red blood cells (fNRBCs) exist in the maternal peripheral circulation and they can be used for molecular analysis eliminating the need for amniocentesis or CVS. The main challenge is that the fNRBCs are rare; 1 per 10^6 to 10^7 maternal nucleated cells. Nevertheless, separation or enrichment of fNRBCs from the maternal circulation is currently achievable in research laboratories using one or a combination of existing methods including magnetic activated cell sorting (MACS) [4], charge flow separation (CFS) [5], fluorescence-activated cell sorting (FACS) [6], density gradient centrifugation [7], immunomagnetic beads [8], ferrofluid suspensions coupled with a magnet [9], and micromanipulation of individual cells [10]. For isolation of fetal cells, MACS and FACS are the methods most frequently used and cited in the literature. These techniques are the best developed, and they thus have the highest potential for success.

MACS cell sorting is based on anti-CD45 (leukocyte common antigen) depletion followed by anti-gamma (γ) positive staining. Cells are labeled with a monoclonal antibody specific to CD45 and attached to a magnetic bead, and are then passed through a magnetic field, at a low flow rate. Presumably all CD45-positive cells are retained in the column, while the CD45-negative cells pass through the column and are collected, thus enriching for the fetal cells. DNA is detected after staining with Vector Blue or DAPI. Variations of this method use CD14 to deplete monocytes and improve the selectivity against maternal cells. CD71, since it is more highly expressed on fNRBCs, can improve enrichment for them [11]. FACS, using anti-γ, relies on the presence of fetal hemoglobin for the positive selection of

fNRBCs. After isolation, mononuclear cells are incubated with anti-γ antibody conjugated with phycoerythrin and stained with Hoechst 33342. Cells fluorescing at both wavelengths are sorted onto slides for manual examination. The fNRBC number is determined on the basis of staining patterns, or by fluorescence *in situ* hybridization (FISH) to detect the X and/or Y chromosome and determine the fNRBC number.

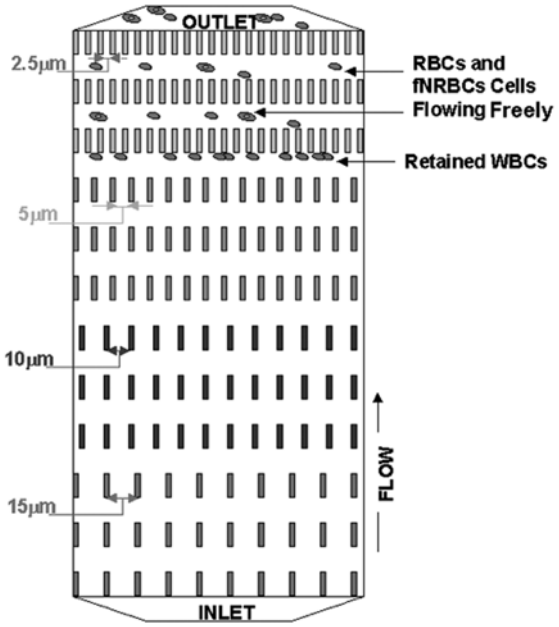

Figure 1: Schematic of device used for fetal cell isolation has varying channel gap widths (15, 10, 5, and 2.5μm). Channel depth, constant over a single device, is 5μm.

While the fetal cell sorting techniques described above have improved over the years, they have yet to become standardized, because of the technical challenges and difficulties in replicating results both within and between different laboratories [11]. FACS and MACS, the most frequently used techniques, require hours for sample preparation, incubation, and the many manipulation steps. Specificity is problematic for either method, due to the lack of a cell specific surface marker/antibody to exclusively detect fetal cells. Experiments using either system require skilled and specially trained laboratory personnel, thereby increasing the cost and limiting test applicability and importantly, availability.

The device under development in our project is designed to address the shortcomings of the existing methods. Sample manipulation is limited to dilution and/or density gradient separation. The fetal cells are separated based on their size and deformation characteristics.

2 METHODS

The device proposed in this study exploits differences in size, structure and deformability between maternal and fetal cells, as a method of separation. The device has four segments of successively narrower channels along the flow axis; these have 15, 10, 5, and 2.5μm spacing (figure 1). This approach was chosen because red blood cells navigate narrow capillary beds, and lymphocytes must migrate from the circulatory system during diapedesis. The first 15μm wide segment served to disperse the cell suspension and create an evenly distributed flow over the entire device, whereas the others were designed to retain increasingly smaller cells. We favored this design over an array of continuous channels without gaps between rows of columns to allow the cells to deform and reform, as they traverse the device. Furthermore if the cell flow is locally slowed or if a channel becomes clogged, a cell can migrate around the affected area (Figure 1). The channel design was laid out with computer-aided design software, and was fabricated on 4" wafers using standard micromachining techniques [12]. All channels have a fixed depth, since the whole wafer is etched at once. After fabrication, each silicon wafer had over a million channels covering a 30mm wide by 60mm long area, with one reservoir on each end. Each reservoir was 30mm by 10mm.

Final devices were molded using polymers such as polydimethylsiloxane (PDMS) (Sylgard-Dow Corning or GE RTV615- General Electric), polystyrene (Sigma-Aldrich), and/or polyurethane (Smooth-On Inc., Easton, IL); these are non-toxic to cells, transparent, handle fluids easily, and can create a high-quality replica of any feature on the wafer down to the sub-micron level.

For this study, devices were either molded in PDMS and sealed with polystyrene tops, or were molded in polyurethane and sealed with polyurethane tops as described previously [12].

Cord blood samples were received from Albany Medical Center using an IRB protocol approved by the New York State Department of Health and were stored at room temperature until used for experiments (within 48 hours). Cord blood was diluted in medium (1:10 v/v) or alternatively, the mononuclear cell layer was isolated from 2mL of blood prior to loading of the device. After Ficoll separation, fNRBCs are in the mononuclear cell layer because they are less dense than mature RBCs. We stained the mononuclear cell layer with a nucleic acid stain (SYTO red, Molecular Probes, Eugene, OR). Next we sought to identify fNRBCs. The buffy coat was stained with fluorescein isothiocyanate (FITC) labeled monoclonal antibody to human hemoglobin F (HbF) (green) (Bethyl Laboratories, Montgomery, TX). Thus white blood cells (WBCs) should fluoresce red only while fNRBCs should fluoresce green and red.

To establish proper flow, the device was first wetted with 2% tetra (ethylene glycol)-dimethyl-ether solution (Sigma Aldrich) in Eagle's MEM and the solution was drawn through the device under vacuum (≅20 inch Hg). The wetting solution was found to have little effect on cell viability for the short-term exposures of these experiments (results not shown). Once the complete device

was completely wetted, the cell solution was introduced. All experiments were performed under a fluorescence microscope with a digital video camera for visualization and recording.

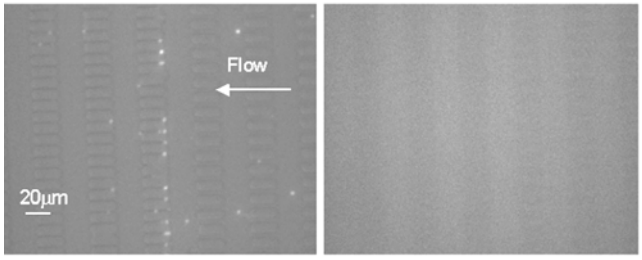

Figure2a: Two images from the same experiment taken with separate imaging filters showing WBCs retained at the start of the 2.5μm wide by 5μm deep channel segment (red filter - left); no HbF positive cells (fNRBCs) were detected in this area (green filter - right) showing that this segment separated the WBCs from the fNRBCs.

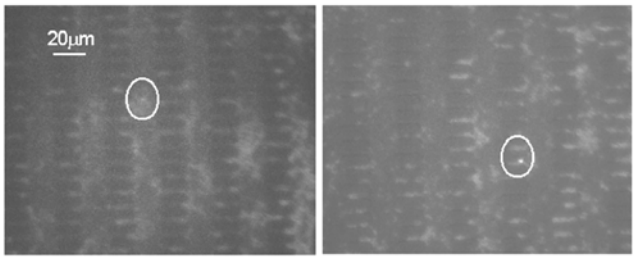

Figure 2b: Fetal cells freely flowing through the device (i.e., not retained) shown in circles.

3 RESULTS

In early experiments, we noted that whole blood or blood diluted in medium 1:10 (v/v) or less obstructed flow even in channels as wide as 10μm due to RBC aggregation (results not shown). However when diluted (1:100 or greater) mature RBCs traverse the entire device unimpeded [12]. At the present time, a one-step centrifugation is required for sample preparation, and only the mononuclear layer is used in the device. This step not only separates the mature RBCs before sample loading, but it also enriches for fNRBCs since mononuclear cells and fNRBCs have similar densities. When the mononuclear layer was tested in the device, white blood cells were retained repeatedly at the start of the 2.5μm wide segment.

While characterizing the migration of human blood cell types, we used goose red blood cells (~12μm) to first mimic human fetal cells (9-13μm), since both are nucleated and they are similar in size. The Griffin Laboratory at the Wadsworth Center provides goose blood samples drawn in heparin when animals are bled for other purposes. Diluted whole goose blood (1:10) in medium was tested in the device and the red blood cells were retained in the 2.5μm wide channels reproducibly demonstrating that the 2.5μm width was a critical dimension for these cells (results not shown). This might have posed a problem if human fNRBCs behaved exactly the same as goose RBCs since maternal WBCs and fNRBCs, would have ended in the same area of the device: the start of the 2.5μm wide segment.

After successful characterization of human whole blood and goose RBCs, we studied heparinized cord blood samples. Cord blood has a higher concentration of fNRBCs relative to the blood from maternal peripheral circulation and thus they are easier to detect. The mononuclear cell layer from cord blood was isolated from 2mL whole blood prior to loading the device. The mononuclear cell layer contains fNRBCs and some mature RBCs. To identify fNRBCs, the buffy coat was stained with fluorescein isothiocyanate (FITC) labeled monoclonal antibody to HbF (green) and SYTO red. Thus fNRBCs should fluoresce green and red (Figure2). Double stained samples were applied to the device, but despite several attempts, fetal cells were not detected at the start of the 2.5μm wide channels with the WBCs as expected from the results using goose blood. FNRBCs, (identified by the double labeling), were observed migrating through the 2.5μm wide channels very slowly compared to adult RBCs, (identified by morphology and absence of both fluors). To investigate this, we designed an overnight experiment to test if fetal cells could traverse the entire device and end up in the outlet reservoir. A cord blood sample from a newborn male was prepared by density centrifugation and was placed in the input reservoir with sufficient medium to preclude drying during the overnight run. Cells were removed from the output reservoir, and their DNA was extracted using a Puregene kit (Gentra Systems, Minneapolis MN), and was tested for X and Y chromosomal sequence by PCR. After agarose gel electrophoresis and staining with ethidium bromide, two bands are present if the DNA is from a male (X and Y), while one band is present if the DNA is from a female. After two rounds of amplification, a very faint Y band was observed in the lane from the sample collected from the outlet tube confirming its male origin with an even more faint X band, suggesting lack of contamination from maternal DNA. Because this method is not the most sensitive, we attempted to use a fluorescence-based identity assay. Another cord sample from a male baby was applied to the device and run 6-8hr. Cells were collected from the output reservoir, DNA was extracted using the Puregene kit, and it was tested using the Cofiler/Profiler kit from Applied Biosystems on an ABI PRISM 3100 Genetic Analyzer. This sensitive assay detects a series of 13 polymorphic markers on various chromosomes and on X/Y, and is used primarily for identity testing, forensics, and to detect maternal cell contamination after CVS. A sample from a male baby will have peaks for both X and Y in this assay. If the DNA was derived from a single individual, the

electropherogram will have 1 (if homozygous) or 2 peaks if the sample is heterozygous at each locus. If the sample is "contaminated", that is if maternal DNA is present, the electropherogram will show 3 peaks for one or more of the markers. Results for one of the cord blood samples are shown in Figure 3. Note one or two peaks per locus demonstrating that the DNA was from a single source, the XY peaks indicate that the DNA was from a male. Furthermore, the peaks are almost the same height indicating the absence of maternal DNA. Otherwise the X would have been significantly higher than the Y peak. There were no peaks in the negative control ruling out contamination in the preparation or the PCR (scans for controls and standards are not shown). Samples were handled with extra care, and contamination from handling was also ruled out (data not shown).

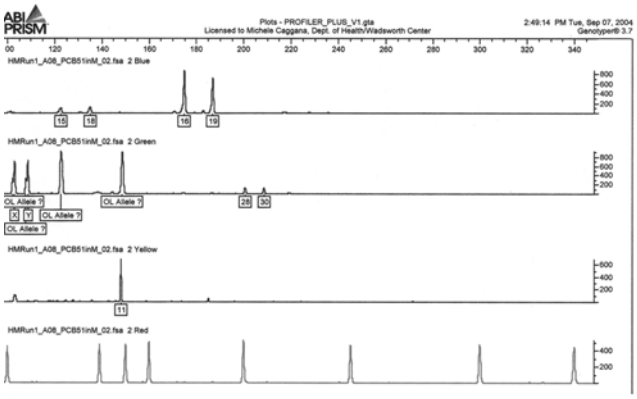

Figure 3: Profiler electropherogram from a cord blood specimen (male baby), tested with the device, note one or two peaks per locus demonstrating the DNA was from a single source (no contamination from maternal DNA), and the XY peaks indicate that the DNA was from a male demonstrating the device's ability to isolate fetal cells (the bottom row is size standard).

4 CONCLUSIONS

We have demonstrated the separation of fNRBCs from maternal cells in cord blood, based solely on size and deformation characteristics. Other than dilution of the blood sample with medium, and density gradient separation, no sample manipulation was required. Future work will determine the device's sensitivity and the minimum maternal blood volume required to run the test. Device fabrication is inexpensive and the operator time is limited to loading and collecting the sample at the beginning and at the end of the test. This device has the potential to eliminate the need for the currently used invasive procedures and to make such testing economical enough to be offered to the entire pregnant population.

ACKNOWLEDGMENTS

The authors would like to thank Dr. David A. Clark, Director of the Children's Hospital at Albany Medical Center, for providing the cord blood samples.

The authors acknowledge the use of the Wadsworth Center's Advanced Light Microscopy Core Facility for the work presented herein.

This material is based in part upon work supported by the STC Program of the National Science Foundation under Agreement No. ECS-9876771.

This work was performed in part at the Cornell Nano-Scale Science & Technology Facility (a member of the National Nanofabrication Users Network) which is supported by the National Science Foundation under Grant ECS-9731293, its users, Cornell University and Industrial Affiliates.

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