Magnetic Cell Separation by Inkjet Printing for Disease Monitoring

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Abstract

The standard analysis technique for cell sorting, flow cytometry, requires centralized facilities such as tertiary Medical Centers. In the U.S. alone, more than 65 million people live in medically underserved areas, many of which need access to diagnostic procedures for proper management of their disease. The goal of this research is to develop a cell sorting technique that can be done in low-resource settings at a decreased cost to the medical organization and the patient. By combining inkjet printing technology and magnetic labeling of cells it is possible to obtain accurate cell counts needing only a regular optical microscope. Mouse CD4+ lymphocytes were attached to micron sized magnetic beads and printed through a modified, commercial inkjet printer. The labeled cells were attached to a glass slide covering a permanent magnet. The cell counts for this study were obtained by use of regular and inverted optical microscopes and NIS-Elements AR imaging software. Comparisons to flow cytometry will be presented. This novel technique may improve upon existing technologies by reducing costs of training personnel, acquisition and maintenance of instrumentation, and time to conduct analysis

Introduction

According to the World Health Organization, as of December 2009 approximately 33.3 million people are currently living with HIV/AIDS worldwide [2]. The data demonstrate that infectious diseases are still a global and national dilemma. Thus, in absence of effective vaccinations, treatment and management for these diseases is paramount. One way of managing diseases such as HIV/AIDS, which specifically attacks CD4+ lymphocytes, involves expensive, bulky equipment that can only be used in specialized clinics and tertiary medical centers. This analysis technique, flow cytometry, can obtain a count of specified cells through immunofluorescent staining. Here we employ a novel method for cell sorting and counting which is inexpensive, portable and has the potential to provide patients with point of care access to properly manage their disease. This new method has the potential to avail patients and laboratories with additional capabilities since it reduces the initial cost of equipment along with maintenance, transportation, and supply costs in comparison to other similar technologies [4].

The cell sorting process is done with a modified thermal inkjet printer that was originally manufactured to be light and portable. Thermal inkjet printing is desirable in cell sorting since it can dependably handle delicate biological material, it is quantifiable in terms of the sample and digital imaging, and provides an automated process that reduces the required skill level of the operator [6,7]. The biological ink used in this printing system consists of a cell mixture that includes micron-sized paramagnetic beads whose surfaces are functionalized with antibodies specific to one cell type. The magnetically labeled beads are collected for evaluation on a modified glass microscope slide that is placed in front of a permanent magnet and the unlabeled cells fall into a gel-coated excess container. An open source imaging software program was used to analyze the images and obtain a cell count.

Heterogeneous cell type mixtures with the same cell concentration as that used in the modified printer along with control samples were analyzed with flow cytometry for validation purposes and to obtain a reference standard.

Experimental Details

The mouse LBRM-33 (clone 4A2) lymphocytes were purchased from ATCC (Manassas, VA) and were derived from radiation induced T cell lymphoma [5]. They were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 0.05 mM 2-mercaptoethanol (Sigma-Aldrich Corp., St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Neonatal Human Dermal Fibroblasts (NHDF) were purchased from Lonza (Basel, Switzerland). The adherent cultures were maintained in Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich), 10% fetal bovine serum augmented by 1% antibiotic/antimycotic (Sigma-Aldrich), and sodium bicarbonate (Sigma-Aldrich) at pH 7.4. Phosphate buffered saline (PBS) (Invitrogen) was used for cell washing. Trypsin (0.25% 1x) with 0.1% EDTA (Sigma-Aldrich) was used for NHDF flask detachment during passaging. The fibroblasts and lymphocytes were cultured in a temperature controlled incubator (Fisher Scientific, USA) with 5% CO_2 at 37°C.

Cell mixtures for the biological ink were prepared in a $1x10^6$: $3x10^6$ ratio of LBRM-33 lymphocytes to NHDF per milliliter. Cell count and viability were assessed by Trypan Blue exclusion. Fibroblast cells were detached using trypsin and harvested via centrifugation at 25°C for 5 minutes and 1000 rpm. The LBRM-33 and fibroblast cell mixture was then centrifuged at 25°C for 5 minutes at 1500 rpm. Pelleted cells were resuspended in PBS isolation buffer with Invitrogen™ Mouse CD4 Dynabeads® (Carlsbad, CA) to create a 1 mL sample of biological ink.

Invitrogen™ Mouse CD4 Dynabeads® were used to separate the LBRM-33 CD4+ lymphocytes from the fibroblasts. The dynabeads are polystyrene microspheres with a 4.5 um diameter and a magnetite core that are coated with the mouse CD4 antibody [1]. Prior to attachment, 25 µL of the beads were washed in 1 mL of isolation buffer which consisted of PBS with 0.1% BSA and 2 mM EDTA. The beads were then resuspended in 1 mL of isolation buffer and added to pelleted cells, and mixed gently. The complete sample was allowed to incubate for 20 minutes at 8°C with slow rotation.

A Hewlett-Packard Deskjet 340 thermal inkjet printer was modified to magnetically sort cells contained within the bio-ink in an automated process that is quantifiable by both sample size and digital imaging (Fig. 1). Modifications to the printer include a lightweight, 6061 aluminum frame with a customized printing platform that holds the permanent magnet and excess container. The printer is capable of printing at an arbitrary angle between vertical (Fig. 2a) and horizontal positions (Fig. 2b). The printing platform is adjustable in the ±x and ±y directions, which allows for flexibility in optimizing the distance between the modified ink cartridge to the permanent magnet. The permanent magnet configuration consists of two Neodymium rare-earth magnets. Both magnets are sintered $Nd_2Fe_{14}B$ Grade 42 with transverse magnetization and a Ni-Cu-Ni triple layer coating for corrosion resistance (Applied Magnets, Plano, TX, USA). The magnet to which the glass slide is mounted is 4" x 1" x 0.5" and has an approximate pull force of 110 pounds. The secondary magnet is 2" x 1" x 1" with about 145 pounds of pull force. The excess container is a 60 x 15 mm petri dish filled with 3 mL 5% gelatin type A (MP Biomedicals, Solon, OH) and 3 mL DMEM media augmented with 10% FBS.

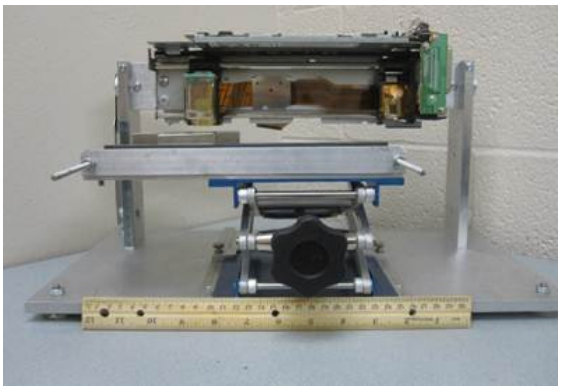

Figure 1. Modified thermal inkjet printer

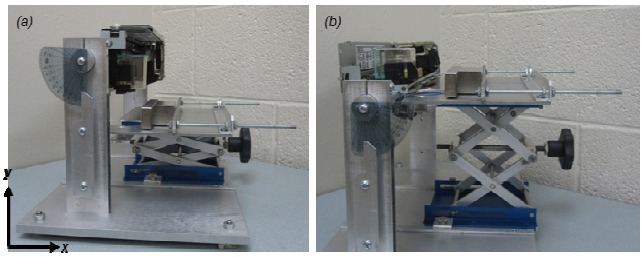

***Figure 2**. Modified thermal inkjet printer (a) Vertical printing position (b) Horizontal printing position*

The ink pens for the printer cartridge were modified to print cells. The original ink and a screen filter above the nozzle area were removed and the cartridge was thoroughly cleaned with ultrasonic agitation in deionized water for one minute. A 0.6 mL conical tube with openings on either end was then inserted into the nozzle pen area. The pen has 50 nozzles with 24 µm diameters and droplet volume of 85 pL [6]. During a single firing of the nozzles, there are 50 pixels printed. Multiplying this by the droplet volume gives an approximate printing volume of 0.43 µL for a single firing of all printer nozzles. During each printout for this study the printer fired 54 times so that the total printout volume was 23 µL. Different sample volumes of the homogenously dispersed bio-ink were tested in order to find the ideal printing capacity for cell sorting. The printed samples were viewed under a Nikon Eclipse Ti inverted optical microscope and 2560 x 1920 pixel images with 0.34 um/pixel resolution were taken using NIS-Elements AR imaging software (Melville, NY).

A printing process for the modified printer was developed and optimized in this study. Prior to printing, the silica microscope slides are thoroughly cleaned with Piranha Solution which is 3:1 sulfuric acid to 30% hydrogen peroxide. This is done in order to clean off any organic molecules that may be attached to the glass surface. Two surface treatments on the glass slides were tested in order to determine the ideal surface on which the labeled cells could be printed without any stray unlabeled cells adhering to the same area. The clean glass slides were coated with either Rain-X™ Original Glass Treatment (ITW Global Brands, Houston, TX) or octadecyl trichloro silane (OTS) (ACROS Organics, NJ) which created a hydrophobic coating. Some of the untreated and treated glass slides were also tested with a layer of 5% gelatin to determine its effect on the printed cells' viability. The treated and untreated glass slides, with or without gelatin, were printed upon in both vertical and horizontal printing positions with variations in the distance between the pen nozzle and magnet. The excess containers were replaced after each printout.

The images taken from the printed sample were used to evaluate cell sorting performance using Image J: Image Processing and Analysis in Java software, open-source software created by the National Institutes of Health (USA) [3]. The images were uploaded in TIFF format with a 100 µm marker used to scale the image. The images were then converted to 8-bit type and the edges of the cells were identified using the software. The threshold of the image was then adjusted and the size maxima was determined. The "particles" were then analyzed and the total cell count in the image was determined.

Flow cytometry using a Gallios Flow Cytometer (Beckman Coulter, Miami, FL) was used to validate the thermal inkjet printing results. LBRM-33 cells ($1x10^6$) were stained with 0.1 µg anti-mouse CD3-FITC, 1 µg anti-mouse CD4-Pacific Blue, or 0.1 µg anti-mouse CD8-PE (all purchased from BD Biosciences, San Jose, CA) for 30 minutes.

Samples were fixed in 200 uL of 1 % formaldehyde following staining (Sigma-Aldrich). Next, flow cytometry analyses were performed on $1x10^6$ cells LBRM-33 with $3x10^6$ fibroblast cells prepared at a 1:3 ratio and stained with 0.1 ug anti-mouse CD4-Pacific Blue and compared to unstained cells.

Results/Discussion

The horizontal printing position was found to be optimal for the printing process and was used in this study. In this position, the printing platform was raised as high as possible while still allowing the printer to remain at 90° and the magnet was approximately 3 cm from the pen nozzle (Fig. 2b). Printouts in the vertical position yielded very little cell collection on the glass slides and mixed cells were found in the excess containers. The surface preparation of the printing substrates was also integral to optimizing the printing process. The Piranha solution effectively cleaned the glass microscope slides and permitted even coating of OTS and Rain-X™. After several printouts, the OTS was determined to be the best surface coating since it allowed the buffer solution to slide off the substrate with more ease while still capturing the magnetically labeled cells. The layer of gelatin on both treated and untreated glass slides was found to have no effect on the labeled cell's viability and its use was thus discontinued. A 10 µL printing volume was determined to be optimal since a single layer of distinct cells was printed on the slides; which is conducive to obtaining an accurate cell count as observed in Figure 3a, where the LBRM-33 lymphocytes are evenly distributed across the image. This figure, taken at 10x magnification, also shows the dynabeads attached to the backsides of the translucent cells. For control test purposes, the cells in the image were compared with images of unmixed , labeled LBRM-33 lymphocytes (Fig. 3b) that were printed in the same manner. Figure 4 shows a scanning electron microscope image, taken 48 hours after printing, and demonstrates that dynabeads attach to the cell membrane and tend to gather on the backside of the cell as they are pulled by the magnetic field of the permanent magnet.

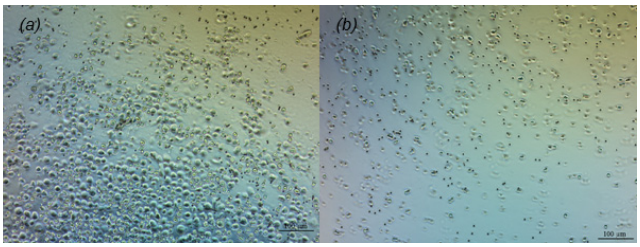

***Figure 3**. Printed cells at 10 µL sample loading volume (a) magnetically labeled LBRM-33 T cells collected on glass slide (1:3 mixed sample) (b) unmixed, magnetically labeled LBRM-33 T cells*

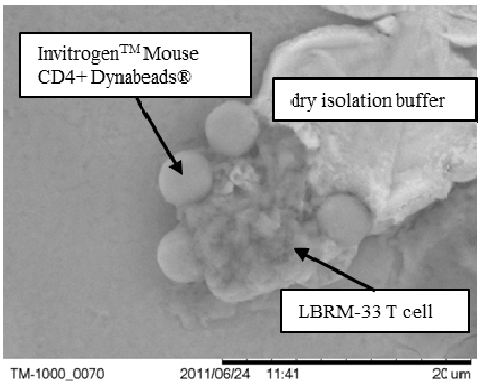

***Figure 4**. SEM micrograph of bead attachment to cells*

In order to determine the volume contained within an image of the printed bio-ink sample at 10x magnification, a control sample consisting of only magnetically labeled LBRM-33 lymphocytes at a concentration of $1x10^6$ cells/mL was printed. Figure 5 shows the images of the control sample with its corresponding adjusted threshold analysis in Image J that were used to obtain cell counts. A volume of 10 µL of the control sample was loaded into the printer pen for each printout. An average cell count of 1450 from the control sample printed images was obtained from Image J. Thus, assuming a homogenous solution, there are about 10,000 cells in 10 µL of the sample which means the volume in a given printed image at 10x is about 1.5 µL. This sets a standard in which each printed image at 10x magnification of a sample containing $1x10^6$ LBRM-33 lymphocytes/mL is approximately 1.5 µL and has about 1450 cells.

An average LBRM-33 T cell count of 1850 in the 10 µL printed images of the bio-ink was obtained from Image J analysis. When compared to the standard, this gives a concentration of about 12300 cells per 10 µL and an overall concentration of $1.23x10^6$ cells per mL. This was consistent with the $1x10^6$ LBRM-33 lymphocytes/mL in the bio-ink mixture.

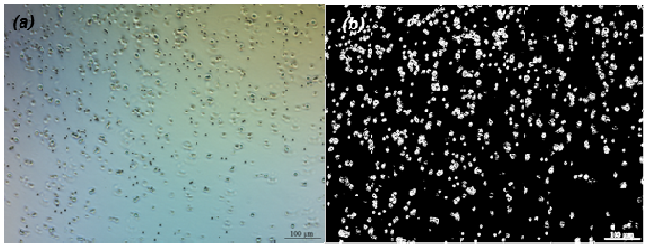

***Figure 5**. Printed images analyzed by Image J (a) unmixed, labeled LBRM-33 lymphocytes (b) corresponding adjusted threshold*

Initial testing with flow cytometry of the LBRM-33 (4A2) cell line verified the presence of CD4+ cells in approximately 93% of the population (Fig. 6). The overall assessment of the stained and unstained 1:3 cell LBRM-33 to fibroblast mixtures show expected, significant differences in size and granularity between the NHDF cells and the LBRM-33 lymphocytes as determined by forward scatter and side scatter analysis using flow cytometry. Figures 7a and 7d demonstrate this and show the gated positions of

each cell type. The NHDF cells, in both the stained and unstained samples, are the larger and more abundant population which appears above the smaller and less granular LBRM-33 lymphocytes. By forward scatter and side scatter analyses, fibroblasts composed 83.4% of the sample while the latter composed 12.3% of the population (Figures 7a and d). Furthermore, 43.3% of the cells gated as lymphocytes in the forward and side scatter plots were CD4+ (Figure 7e) as compared to unstained cells used as negative controls (Figure 7b). In contrast, the majority of the fibroblast-gated population were not CD4+ (Figure 7f) as compared to the unstained negative control fibroblast population (Figure 7c). Figures 7c and 7f show a slight difference in the peak positions for the NHDF cells in the stained and unstained samples. Ideally, these peaks should be identical but peak shifting may be due to nonspecific staining and the possibility of lymphocytes being included in the fibroblast gating.

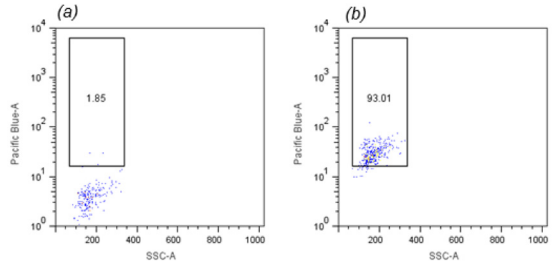

***Figure 6**. Flow cytometry results for LBRM-33 lymphocyte (a) unstained (b) stained CD4 with 1 µg Pacific Blue*

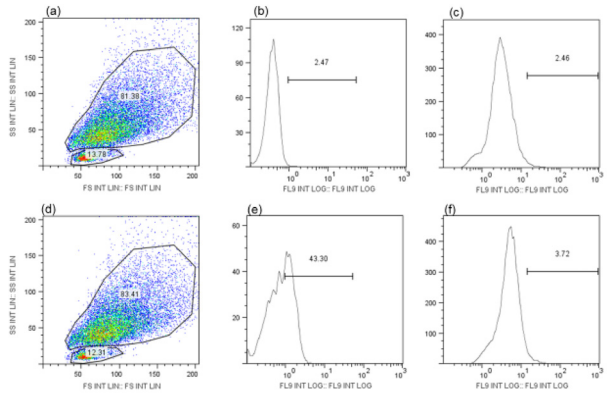

***Figure 7.** Flow cytometry results for 1:3 cell mixture (a) unstained population gating (b) unstained LBRM-33 lymphocytes in mixture (c) unstained NHDF in mixture (d) stained population gating (b) stained LBRM-33 lymphocyte in mixture (e) stained NHDF in mixture.*

Summary/Conclusion

The goal of this study was to create an inexpensive, portable alternative to flow cytometry for low resource settings. This was achieved through the modification of a commercially available thermal inkjet printer and creation of an optimized printing process to sort cells. The modifications include low-cost materials such as a stand made out of a common aluminum alloy, commercially available neodymium magnets, and open source imaging software. The printing process was optimized to be simple and efficient so less training would be necessary to the operator in comparison to flow cytometry. The biological ink used in this study was a 1:3 ratio of LBRM-33 lymphocytes to neonatal human dermal fibroblasts. Although this mixture would never occur naturally, the significant difference between cell types allows for a more accurate analysis; at least in these pilot experiments. An average cell count of about $1.2x10^6$ LBRM-33 lymphocytes/ mL was obtained from Image J analysis of mixed sample printouts based on the known concentration of control samples. Flow cytometry results verified the presence of the CD4+ protein on the LBRM-33 lymphocytes' membrane along with demonstrating a significant size difference between the two cell types. This validated the use of protein specific attachment of magnetic microspheres to the lymphocytes for cell sorting. In its first stages, this prototype for a simplified alternative to flow cytometry in medically underserved areas appears to have great potential for the future.

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