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Design and fabrication of a microfluidic device for separation of lymphocytes for use as part of a radiation biodosimeter

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Abstract

A radiation biodosimeter is a measure of biological response to radiation that can help determine the exact dose received by using biological markers or radiation-damaged chromosomes using a blood sample as a surrogate tissue. Today, many researchers are interested in micro-technology known as microfluidics which is a device that deals with the theory of fluid flow in sub-millimeter sizes. This research demonstrates the design and fabrication of a microfluidic device for separation of lymphocytes from whole blood using a centrifugal technique. The device is made of a circular PMMA (Sumipex) sheet with a diameter of 90 mm and a thickness of 5 mm, consisting of 12 chambers, each a 3 mm deep hexagonal cavity with a capacity of 120 µL and a straight channel 20 mm long. Efficacy testing was performed by loading 120 µL of whole blood into a chamber and centrifuging at 3000 rpm for 5 min. A yellow layer was removed and overlaid on the Ficoll solution (1.077 g/mL) and centrifuged at the same speed. The peripheral blood mononuclear cells were then removed and stained with trypan blue before counting in the hemocytometer. The results show that the developed microfluidic device is able to separate lymphocytes efficiently, which is used as part of a radiation biodosimeter.

Keywords: Microfluidics, Lab-on-a-disc (LOD), Lymphocyte, Density gradient, Biodosimeter

1. Introduction

The use of nuclear technology has increased dramatically worldwide, especially in the electric power industry, research, and human health application. Despite the strict rules and safety measures, nuclear accidents or unplanned radiation exposure can happen. In the event of a radiation emergency, biological dosimetry is necessary for estimating the amount of radiation exposure of individuals such as exposed workers and the public. It can be performed regardless of the magnitude of the accident. In a large-scale accident, the data obtained with this technique may help differentiate the people exposed to radiation from those who are not for further appropriate treatment. Biological dosimetry includes different cytogenetic assays, including dicentric chromosomes, premature chromosome condensation, micronuclei, and fluorescent in situ hybridization, which are used to assess the dose absorbed by the exposed individuals. Cytogenetic dosimetry is recognized as a valuable method for estimating radiation doses as it fills a gap in physical dosimetry, especially for exposed individuals who do not wear dosimeters [1]. Biological dosimetry assays are generally performed on lymphocytes in the bloodstream and have been proven successful in routine use [1]. Whole blood consists of four main components: plasma, red blood cells (RBCs), white blood cells (WBCs), and platelets. WBCs are composed of neutrophils, eosinophils, and basophils collectively known as polymorphonuclear (PMN) cells, while lymphocytes and monocytes are collectively referred to as peripheral blood mononuclear cells (PBMCs) [2]. Peripheral blood lymphocytes have a significant advantage over other cellular systems as they circulate throughout the body. Therefore, they are at the same stage of the cell cycle at both time of exposure and time of blood draw, resulting in the same amount of DNA and the same type of chromosomal damage. Centrifugation of blood in a density gradient medium such as Ficoll solution is an effective technique for separating and sorting blood cells into different layers according to their density differences [3].

The development of biological dosimeters that can perform rapid analysis is therefore of paramount importance in radiation emergencies. Key requirements for equipment used in a radiation emergency are low manufacturing cost, long shelf-life, and usability with minimal training by semi-skilled operators. Additional requirements are to use a minimal amount of analyte, and the blanks and calibration samples can be run under identical conditions. At present, efforts are made to develop devices for these purposes based on microfluidic technology which manipulates fluids in structures on a size-scale of micrometres. There are two main platforms of microfluidics: lab-on-a-chip (LOC) and centrifugal microfluidics or lab-on-a-disc (LOD) [4]. The advantages of both platforms are lower total cost, less material and reagent usage, faster response times due to lower fluid volume and diffusion distances, portability,

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and programmability [5-7]. The centrifugal microfluidic platform involves three forces-the centrifugal force, Coriolis force, and Euler force-to manipulate liquid flow on the disc [8]. As a result, the LOD has many advantages, such as facilitating handling multiple samples concurrently, separating the sample components according to their density, and allowing the liquid to flow in the pipe without direct contact with the external hardware [5]. The successful implementation and use of the LOD system depend on a cheap and reliable production of circular disc disposable products. Typical materials used are polycarbonate, poly (methyl methacrylate) (PMMA), cyclic olefin polymer (COP), polydimethylsiloxane (PDMS), and polyurethane [5].

Several MF devices have been developed recently for biological application [9], especially for cell separation, classification, and sorting [10-15]. Such devices can be classified as two main types: separation-based methods that utilize the differences between target cells and other cell types in size [10, 11] or inertial devices [12-14], and flow-cytometric methods which allow direct sorting of target cells without sample separation [15]. Although these methods result in the efficient separation of target cells, most of the developed devices are complex in terms of fabrication and functionality. Therefore, this research is interested in developing a MF device using a separation-based approach for isolation of lymphocytes from whole blood, which stands out as a simple device and uses basic laboratory equipment in both invention and operation.

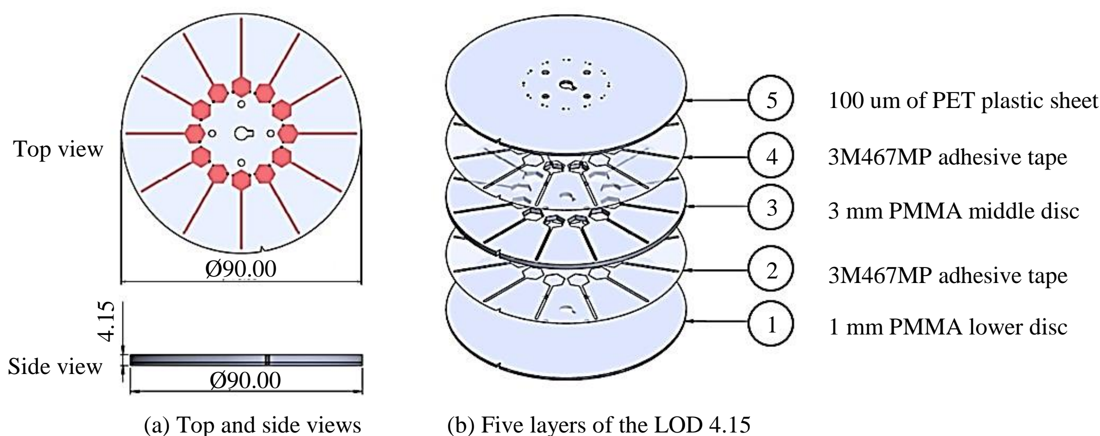
The fabrication of a microfluidic device for use as a biological dosimeter can be divided into smaller components according to the analytical steps. In this study, a device capable of separating lymphocytes from whole blood using the LOD platform was developed according to their density-based separation, which requires centrifugation. The efficacy of the MF device was tested with real lymphocyte isolation compared with conventional separation using microcentrifuge tubes. The developed MF device is expected to be integrated with the second device involving gamma-H2AX foci analysis in lymphocytes for further radiation dose assessments, which is being developed in our laboratory.

2. Materials and methods

2.1 Design and fabrication of LOD

This experiment was conducted at the Thai Microelectronics Center (TMEC), Chachoengsao, Thailand. The LOD models were designed using the SolidWorks drawing program. A laser cutting and engraving machine (HY-6040S, HYLAX Co., Ltd.) was used to create channels. Disc centrifugation was performed using a benchtop centrifuge (WiseSpin CF-10, Daihan Scientific, Korea).

LODs were fabricated with a 90 mm diameter from acrylic or PMMA sheets (Sumipex (Thailand) Co., Ltd.) (Figure 1(a)). The final disc design consisted of 5 layers (Figure 1(b)) arranged from bottom to top as follows: a 1-mm thick PMMA bottom, double-sided adhesive tape (3M467MP, USA), a 3-mm thick PMMA middle with hexagonal chamber and 20 mm long straight channel, double-sided adhesive tape, and a 100-µm thick polyethylene terephthalate (PET) sheet. Each layer was joined by a plate holder and compressed by a hand press machine. Each disc contained 12 reaction chambers, each with a capacity of 120 µL.



(a) Top and side views (b) Five layers of the LOD 4.15

Figure 1 Final LOD design

Prior to the final LOD design, the experiment focused on two key elements to enable the separation of lymphocytes from whole blood: the design of the middle plate to store whole blood and RBC samples, and the design of the top plate to allow the PBMC layers to be taken from the LOD after separation. The LOD design and fabrication were divided into PMMA middle disc design and top disc design.

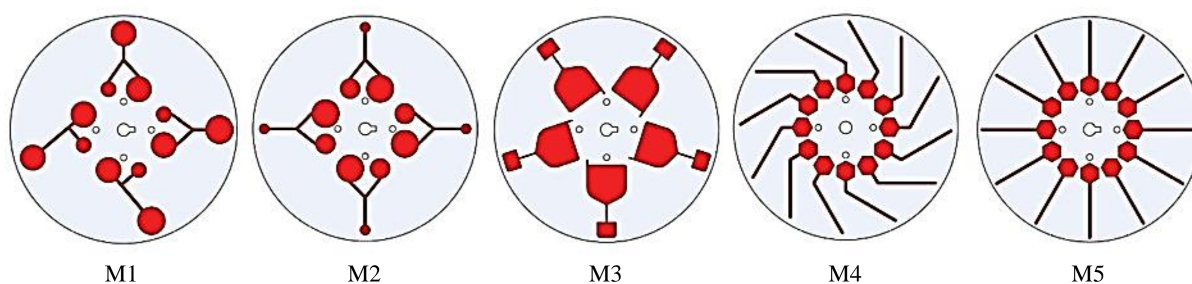


Figure 2 Five patterns of PMMA middle disc designs

2.1.1 PMMA Middle disc designs

The middle disc layout was designed and fabricated into five patterns, as shown in Figure 2. Each pattern differed in chamber shape and configurations, i.e., circular chambers with Y-shaped channels (M1 and M2), cup-shaped chambers (M3), and hexagonal chambers with 45-degree inclined (M4) and straight channels (M5). Initially, efficacy tests were performed for lymphocyte separation after centrifugation. The speed of centrifugation was adjusted according to the basic method of blood separation. After that, the optimum centrifugation speed and time were obtained at 3000 rpm for 5 min.

After the M1 and M2 centrifugation, the blood samples were confined to the upper chamber. For M3 the separation of blood layers was clearly visible, but the upper chamber was too wide and the PBMC layer was not visible. For M4, the separation of blood layers was clearly visible, but because of the inclined channels it was difficult to estimate the PBMC layer, while for M5 it was more noticeable. Therefore, M5 was the best design to separate the PBMC layer and was chosen for the next step in the top disc design.

2.1.2 Top disc designs

The design of the top disc, which is used to remove lymphocytes from the disc, consisted of 4 patterns as shown in Figure 3. T1 was a normal PMMA disc with inlet and vent holes. T2 was modified from T1, and the suction hole was added. T3 was developed from T2 by adding a rectangular channel for easier removal of the PBMC layer. T4 had the same pattern as T1 but was made of a 100 µm thick PET plastic sheet. As a result of testing for lymphocyte suction from all four models using a 500 µL insulin syringe with needle, it was found that the T4 pattern was appropriate for sucking the PBMC layer up. Therefore, T4 was chosen for fabrication of the LOD.

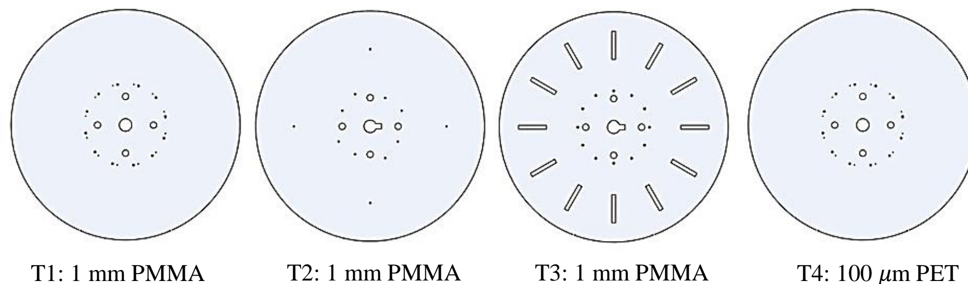


Figure 3 Four patterns of top disc designs

2.2 Blood sample collection and the LOD efficacy testing

Blood collection was approved by Kasetsart University Research Ethics Committee (KUREC). Written informed consent was obtained from six healthy donors prior to blood collection.

The average number of lymphocytes required for gamma-H2AX measurement is approximately 6 x 10^5 cells [16], which is 100-200 µL of whole blood. According to a standard method performed in 50 mL tubes, the whole blood must be diluted 1:1 with 2% PBS prior to overlaying 35 mL on 15 mL of Ficoll solution (1.077 g/mL, Merck, Germany). For the LOD, this method was minimized to reduce time, reduce sample volume, and increase efficiency. Therefore, it was modified by starting with 120 µL of whole blood in a hexagonal chamber on the LOD and centrifuged at 3000 rpm for 5 min. After centrifugation, the 60 µL yellow layer was removed with a 500-µL insulin syringe and overlaid on the pre-filled Ficoll solution on the LOD at a 1:1 ratio and then centrifuged at the same speed. After centrifugation, 10 µL of PBMCs or buffy coats were removed and stained with trypan blue before counting the lymphocytes in a hemocytometer. The morphology of lymphocytes was observed by an inverted microscope (Motic Images Plus 3.0, Hong Kong). The experiments in Eppendorf tubes were performed in parallel to compare the results with the LOD trials. Diagrams of the LOD and Eppendorf experiments are shown in Figures 4 and 5, respectively.

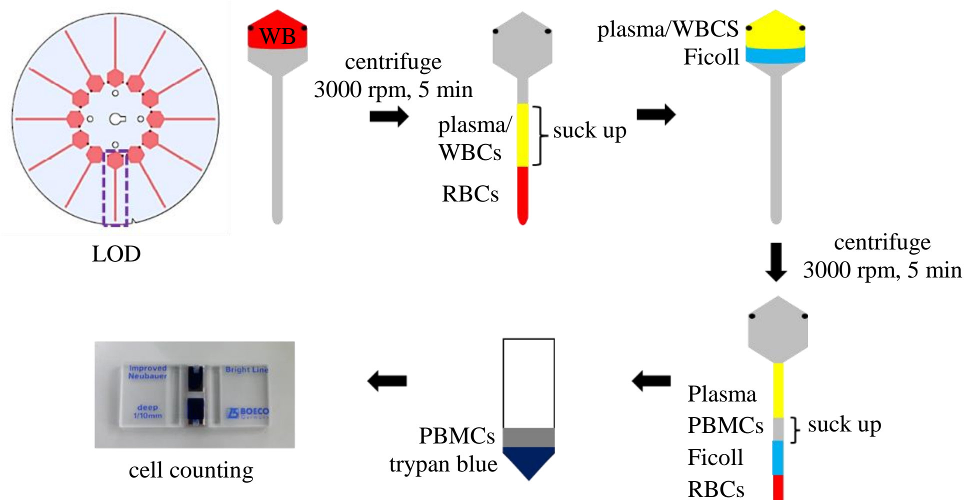


Figure 4 Diagram of the LOD experiment

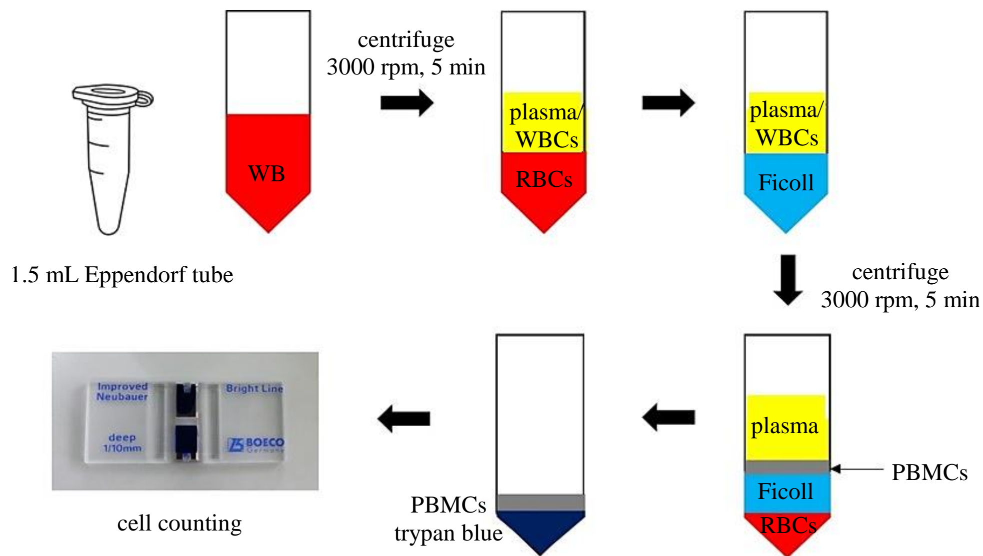
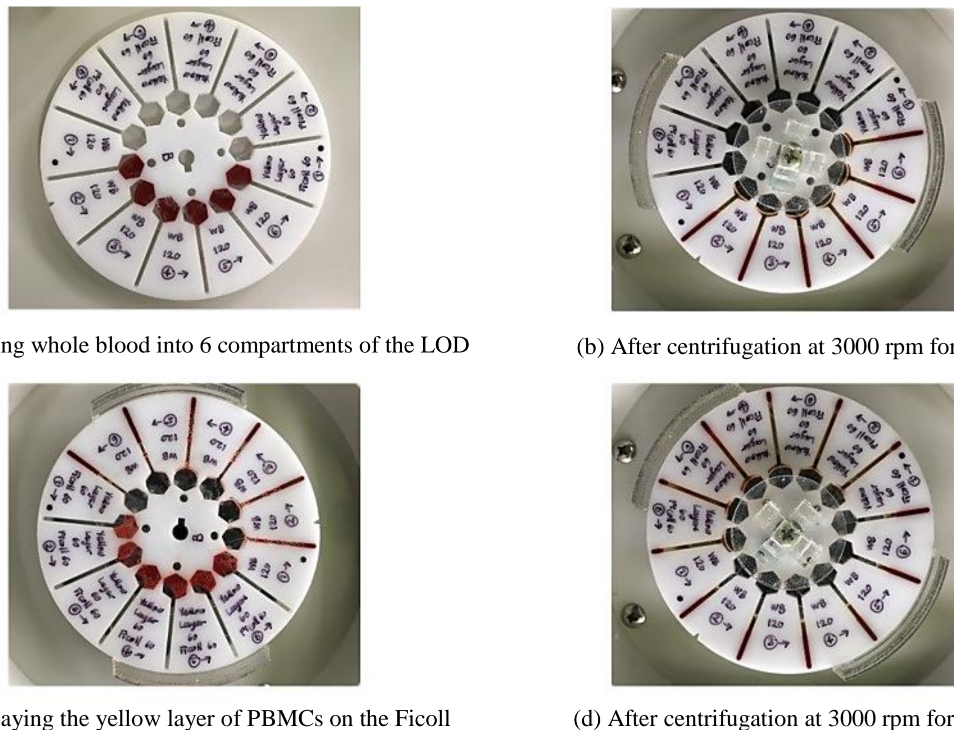


Figure 5 Diagram of the Eppendorf experiment

3. Results and discussion

In designing the LOD, particularly for the middle disc in our study, it was necessary to consider the persistence of the RBCs as well as the location for the removal of the PBMC layer after centrifugation. The model used in the test was divided into five patterns. From the preliminary testing of those patterns by centrifuging the blood sample and observing the stratification, it was found that the hexagonal design was the easiest to assess the location of the PBMC layer. As seen in Figure 6(a), 120 µL of undiluted blood was added to the six compartments on the LOD and centrifuged at 3000 rpm for 5 min. After centrifugation, stratification was observed. RBCs and a clear yellow layer were clearly visible, as shown in Figure 6(b). Then 60 µL of the clear yellow layer was transferred using a 500 µL insulin syringe with needle into the LOD chamber where 60 µL Ficoll solution was prepared (Figure 6(c)). It was then centrifuged at 3000 rpm for 5 min. After centrifugation, a new insulin syringe was used to carefully remove an approximately 5 mm layer above the RBCs with a volume of 10 µL (Figure 6(d)).



(a) Loading whole blood into 6 compartments of the LOD

(b) After centrifugation at 3000 rpm for 5 min

(c) Overlaying the yellow layer of PBMCs on the Ficoll

(d) After centrifugation at 3000 rpm for 5 min

Figure 6 Efficacy test of the LOD

The centrifugation speed of 3000 rpm was determined by calculations based on the standard separation of lymphocytes performed in a 50 mL centrifuge tube using 35 mL of undiluted blood and centrifuged at 400x*g*. The centrifugation speed calculated for LOD with a diameter of 90 mm is approximately 3200 rpm when the particles are at a distance of 30-40 mm from the center of the LOD. The separation of PBMCs from RBCs occurs by the different centrifugal forces between the blood components. The centrifugal force is expressed in the following equation [17]:

$$F = m\omega^2 r \quad (1)$$

where F = magnitude of the centrifugal force; m = effective mass of the particle; ω = angular velocity (rad/s) and r = distance of the particle from the axis of rotation.

Based on the density of lymphocyte (1.070 g/mL) and RBC (1.10 g/mL), their centrifugal forces in the LOD at the operational speed of 3000 rpm calculated from Equation (1) are approximately 0.4435 and 0.4555 N, respectively when the particles are at a distance of 30-40 mm from the center of the LOD. In addition to centrifugal force, the separation of lymphocytes from whole blood is facilitated by Ficoll density gradient centrifugation that takes advantage of the density differences between PBMCs and other constituents in the blood sample. Therefore, cells are distributed in layered solutions according to the difference in their density.

After the second centrifugation, a distinct stratification of RBCs and PBMCs was observed, as shown in Figure 7. A layer approximately 5 mm above the RBCs was removed, stained with trypan blue, and the cells were counted using a hemocytometer. The images of the lymphocytes counted in the LOD test were very similar to those obtained in the Eppendorf tube test (Figure 8). As tested with trypan blue exclusion assay, a viable cell had a clear cytoplasm whereas a nonviable cell had a blue cytoplasm.

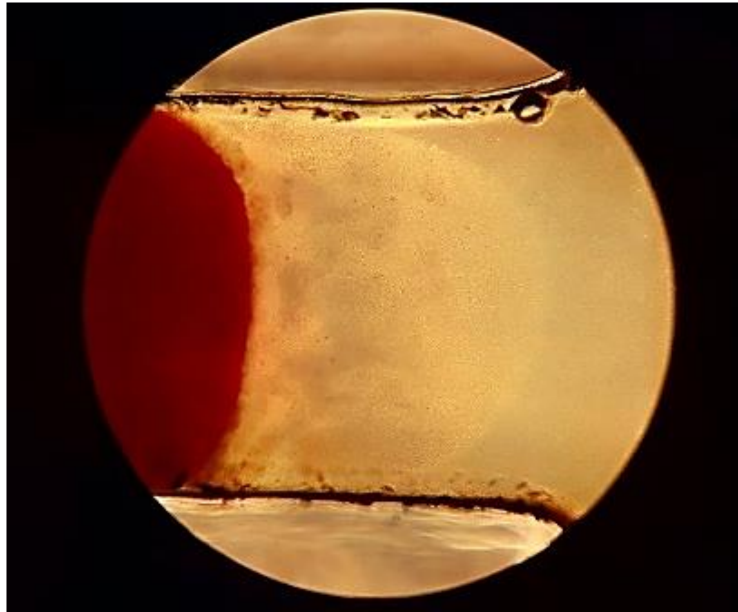
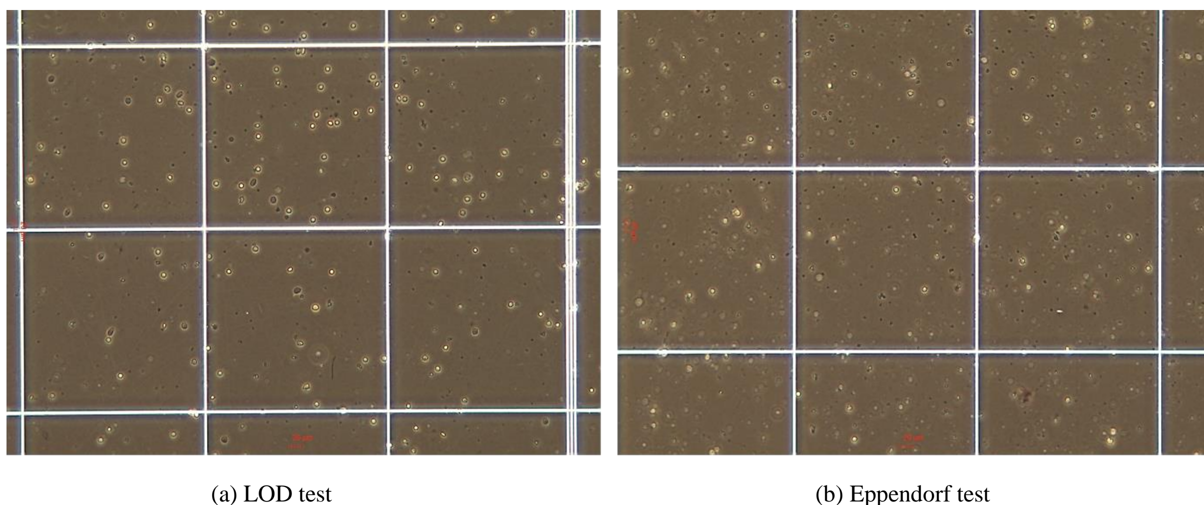


Figure 7 Milky layer of PBMCs observed on the LOD by inverted microscope (10X)

Based on the number of lymphocyte 6 x 10^5 cells required for use in the gamma-H2AX foci analysis, experiments were performed to optimize the operational parameters to ensure that the number of isolated lymphocytes was similar to the standard method. At first, 60 µL of whole blood was overlaid on 60 µL of Ficoll (1.077 g/mL) and centrifuged for 10 min, but the PBMC layer was unclear, and the number of lymphocytes was not enough for gamma-H2AX analysis. Then, the experiment was changed by adding 120 µL of whole blood into the chamber, centrifuging, and extracting only the yellow layer, which was overlaid on Ficoll solution. This test took 20 min and the PBMC layer was clearly observed. After staining with trypan blue, the lymphocytes visualized under an inverted microscope were round and clear with a diameter of 15-20 µm (Figure 8). The results of counting the average number of lymphocytes are shown in Table 1. The average lymphocyte count based on five individual tests was 1.6 x 10^6 cells/mL, which was similar to the average count from the Eppendorf of 1.3 x 10^6 cells/mL, indicating that the LOD was as effective as the standard separation in Eppendorf tubes.



(a) LOD test (b) Eppendorf test

Figure 8 Lymphocyte counting using a hemocytometer and visualizing under an inverted microscope (10X)

Table 1 The average number of lymphocytes isolated from five individual tests in the LOD and Eppendorf tubes

Number of lymphocytes (cells/mL)			
#	**LOD**	**#**	**Eppendorf**
1	1.92 x 10^6	1	1.24 x 10^6
2	1.22 x 10^6	2	1.36 x 10^6
3	1.53 x 10^6	3	1.04 x 10^6
4	1.59 x 10^6	4	1.30 x 10^6
5	1.57 x 10^6	5	1.33 x 10^6
Average	1.56 x 10^6	Average	1.25 x 10^6

When comparing the results with similar recent studies, such as that of Kim et al. [18], a new type of microfluidic cell counter was developed that enabled the enumeration of the low-abundant residual white blood cell (rWBC) count in leukodepleted blood products, which is beneficial for the safety of blood transfusion. Yu et al. [3] developed a centrifugal microfluidic chip capable of sorting immune cells from whole blood. Kinahan et al. [19] demonstrated a developed LOD cartridge for the isolation of leukocytes from whole blood using centrifugo-pneumatic siphon valves. There have also been many microfluidic platforms for cellular differentiation reported in the literature over the past decade [20-29], but most of them were found to be complex systems. Furthermore, very few studies have been designed to be applied directly to blood samples, especially for use in biodosimetry applications. This research is one of the studies that have developed the LOD for lymphocyte separation, which has the advantage of simple fabrication and operation, only a low-cost single purpose centrifuge is needed. Lab centrifuges are quite complex, delicate and expensive. Our simple device which is made from PMMA sheets bonded with adhesive tape has a low production cost. They can be produced in large batches and can be operated with little training. The developed LOD can be integrated with the gamma-H2AX microfluidic device being developed in our laboratory. It is therefore ideal for radiation emergencies that require accurate results with fast analysis. In addition, white blood cell isolation is an important sample preparation step for many biological tests, and this work is expected to benefit other applications related to the use of white blood cells.

4. Conclusions

Centrifugal microfluidic discs, or LODs, for the separation of lymphocytes were designed and fabricated with hexagonal chambers and straight channels. The developed LODs can separate six whole blood samples simultaneously in 20 minutes, each containing 120 µl. The greatest advantage of using a LOD is its ease of fabrication and portability. Small blood samples and reagents can be loaded into the LOD using a simple micro-pipette. Lymphocyte separation can be performed using 3000-rpm centrifugation at room temperature and atmospheric pressure. The mean WBC count obtained from the LOD was 1.6 x 10^6 cells/mL. This WBC yield is quite adequate for biodosimetry using gamma-H2AX foci analysis.

5. Acknowledgements

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6. Conflicts of interest

The authors declare no conflict of interest.

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