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微流體碟盤系統用於母血中胎兒細胞純化進行非侵入式產前檢測

Isolation of Fetal Cells in Maternal Blood for Non-invasive Prenatal Diagnosis Using a Microfluidic Disk System

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Isolation of Fetal Cells in Maternal Blood for Non-invasive Prenatal Diagnosis Using a Microfluidic Disk System

本論文係許展毓君（學號：R04543026）在國立臺灣大學應用力學研究所完成之碩士學位論文，於民國 106 年 7 月 20 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

在台灣每年大約有 20 萬名新生兒,其中大約有 4 萬名孕婦是屬於高齡產婦(年紀大於 35 歲),因此產前檢測變得相當重要。侵入型產前檢測,如:羊膜穿刺、絨毛膜取樣等技術,雖然是目前公信的標準,但卻伴隨著流產的風險以及對母體的傷害。近年來非侵入式產前檢測受到許多團隊的矚目,如: 胎兒游離 DNA 檢測、從母血中分離胎兒細胞等技術,胎兒游離 DNA 因具有偽陰性與偽陽性的機率,因此即使檢測出有基因疾病的可能性,仍然要做侵入式產前檢測作確診。另外,由於胎兒細胞在母血中含量非常稀少,以及胎兒細胞表面抗體的不明確,所以必須發展一高靈敏度、高產量以及自動化的技術。

本研究提出一微流碟盤系統利用密度離心方式分離胎兒細胞、螢光辨別系統以及單一細胞抓技術與後端基因分析的建立。此研究包含一微流結構碟盤進行分離細胞、一具有微流結構收集管進行細胞收集與染色、收取到的細胞用於進行後端的基因分析。可拆卸式微流收集管不僅方便染色時進行混合的動作,也方便離心步驟結束的檢體取出做螢光辨識以及後端的基因分析,且自動化的流程可以減少人為操作變異。系統經由滋胚層癌化細胞株(JEG-3)來驗證,將定量好的細胞加進 4 毫升的健康人血液中,以模擬胎兒細胞在母血中的情境,而在經過整個實驗流程後,包含梯度密碟盤分離,以及在微流收集管進行 CD45、HLA-G、cytokeratin、Hoechst 33342 的螢光染色,並以螢光顯微鏡進行判讀,最後使用單一細胞抓取技術取得 5 到 7 顆高純度、高準度的目標細胞,以進行全基因放大、聚合酶連鎖反應、短縱列

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重複序列驗證。實驗結果顯示此微流碟盤系統回收率約 80%(79.5±3.5%)，取五段不同的染色體(SRY, G3PDH, chromosome 3, chromosome 11, and chromosome 13)進行聚合酶連鎖反應驗證，而結果顯示每個片段均有成功放大出來，另外，還更進一步選用六段不同的基因(D19S433, vWA, TPOX, D18S51, D5S818 and FGA)做短縱列重複序列驗證，結果顯示所放大出來的基因跟參考的細胞株一樣，並與非孕婦健康人的基因不同。因此，本研究發展一微流碟盤系統搭配自動化操作平台回收稀少之胎兒細胞，並進行後端全面的基因分析，希望在將來跟醫生收取檢體可提供順暢的檢體處理流程，以及孕婦產前檢測可信賴的方法之一。

關鍵字: 胎兒細胞；密度梯度離心；微流體

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Abstract

Traditional prenatal genetic diagnosis relies on invasive procedures such as amniocentesis and chorionic villus sampling in order to obtain the fetal cells. However, these procedures may lead to abortion in about 1 in 100 to 500 incidences. Hence, non-invasive prenatal diagnosis, such as cell-free DNA and isolation of circulating fetal cells, has gained prominence in recent years. The ability to harness fetal cells is extremely attractive, if successful, they have the potential to enable comprehensive fetal diagnosis. However , these cells are extremely rare in maternal circulation, rendering development of a sensitive, robust, and automated technology a challenge.

In this thesis, a novel microfluidic device using density-based separation, fetal cells enrichment and multi-step process of immunofluorescence (IF) staining were presented. The device contains a centrifugal disk for enrichment of target cells and a tube-based collector, which can easily wash IF dye and enable sample removal after the separation process. The JEG-3 cell line was used to verify the performance of the device with cells spiked into the blood of healthy donor labeled with HLA-G, cytokeratin, and Hoechst nuclear dye after enrichment. A microfluidic single cell pick technique was applied to retrieve 5 to 7 target cells to mimic the rarity of fetal cells in maternal circulation. Whole genome amplification (WGA) was used on retrieved single cells to amplify all genes followed by polymerase chain reaction (PCR) and short tandem repeat (STR). Results

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show that the recovery rate of the device reaches nearly 80% (79.5±3.5%). Five different chromosomes (SRY, G3PDH, chromosome 3, chromosome 11, and chromosome 13) successfully confirmed that the amplified genes were from JEG-3 target cells. The STR profiles of PCR amplicon proved that they originated from JEG-3 target cells with the X-Y chromosomes, as distinguished from non-pregnant woman genes with the X-X chromosomes. Taken together, the centrifugal microfluidic system presented should enable successful retrieval of rare fetal cells in maternal circulation towards non-invasive prenatal diagnosis.

Keywords: Fetal cell；density gradient separation；microfluidics

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Abbreviated table

1. CTC: Circulating tumor cell
2. IF: Immunofluorescence
3. RBC: Red blood cell
4. DNA: Deoxyribonucleic acid
5. cffDNA: Cell-free DNA
6. DGC: Density gradient centrifugation
7. DGS: Density gradient solution
8. FACS: Fluorescent activated cell sorting
9. FISH: Fluorescence in situ hybridization
10. G3PDH: Glycerol-3-phosphate dehydrogenase
11. MACS: Magnetic activated cell sorting
12. NIPD: Non-invasive prenatal diagnosis
13. PCR: Polymerase chain reaction
14. SRY: Sex determining region Y
15. STR: Short tandem repeat
16. WGA: Whole genome amplification
17. RCF: Relative centrifugal force
18. RB+EDTA: Running buffer+ Ethylenediaminetetraacetic acid
19. PMMA: Polymethylmethacrylate
20. PP: Polypropylene
21. MLPA : Multiplex ligation-dependent probe amplification

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Chapter 1 Introduction

1.1 Clinical relevance of fetal cells in maternal blood

For many years, prenatal diagnosis is important to enable pregnant women to have knowledge of genetic issues of the fetus, such as trisomies 21, 18, 13 and monosomy X. Fetal cells have found existing at maternal blood since 1893 by Georg Schmorl[1]. If the intact fetal cells were identified, they contain whole genome fetal DNA uncontaminated by maternal DNA which can be used for genetic testing. However, fetal cells are very rare in the maternal blood, hence their isolation and subsequent analysis difficulty to realize their value.

Invasive prenatal diagnosis, such as the chorionic villus sampling (CVS) and amniocentesis, not only accompany with 0.5~2% risk of miscarriage in about but also possess risk to the mother, so noninvasive methods are preferred over invasive approaches[2, 3]. The abdominal ultrasound exam, cell free-fetal DNA and fetal cell within maternal blood are the common methods of noninvasive prenatal diagnosis. The abdominal ultrasound exam is applied to evaluate the congenital disorder or abnormalities of fetus by image analysis, but it may come out with false positive rate for trisomy 21 in about 4~6% and no provide genetic diagnosis[4]. There two main difficulties in the detection of fetal abnormalities procedure by fetal DNA in maternal blood very difficult. One is that the low concentration of fetal DNA, about 3~6% of total plasma DNA in

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maternal blood, and the other one is that all of fetal DNA are fragmented[5]. Thus, to develop a reliable technology of enrichment of fetal cells within maternal blood still plays an important role in noninvasive prenatal diagnosis.

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1.2 Technologies for fetal cells enrichment

Although much effort was made to continuously improve the technology for enrichment of fetal cells, their rarity still possess a great challenge. Thus, the enrichment and detection methods require high sensitivity, specificity and capable to handle large volume of blood. From the past few years, there have been many noninvasive prenatal diagnosis methods to enrich fetal cells from maternal blood. These technologies for enrichment are divided into two main categories, one bases on physical properties such as size, density and electric charge, and the other one bases on biological properties such as surface protein expression.

Physical properties can isolate fetal cells without any labeling. Size-based filtration using microfluidic device with many small pillar structure, which based on the difference size and deformability between fetal cells and other non-fetal cells[6, 7]. R. Huang et al.[8] built a device with a 2.5μm wide and 5μm deep channel to isolate fetal nucleated red blood cells, which have range in diameter from 9 to 12μm. The ScreenCell filtration devices (ScreenCell Inc.) composed a filtration tank, a microporous filter, and a nozzle can be used to isolate and sort rare cells by size[9-11]. Bøyum established a density gradient separation that is a common method to remove a great number of red blood cells only by a centrifugation process at initial step when received whole blood sample[12]. Different kinds of DGS such as Ficoll-Paque PLUS or Percoll is applied to separate

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different density of cells. Daniel E Campton et al[13], Heather J. Chalfin et al.[14] and Amy M. Breman et al.[15]used a novel device called the Accucyte[16] (RareCyte Inc.) which using density-based enrichment, automated immunofluorescence staining, and digital microscopy with image analysis to identify rare cells.

Biological properties on surface antibody-antigen specificity of cells can be used to separate various cell populations. Magnetic-activated cell sorting (MACS) was applied for either positive selection or negative selection based on different expression of antibody-antigen properties and the method was first found by Stefan Miltenyi et al.[17] at 1989. Xiao Xi Zhao et al.[18, 19] used different fetal specific antibodies to separate fetal cells from maternal blood. Fluorescence-activated cell sorting (FACS) can separate each cell based on the different concentration of antigen on cell's surface and fluorescent characteristics of each cell. After Len Herzenberg[20, 21] applied the FCAS technique to separate fetal cells, more teams are using this device to isolate fetal cells[22, 23]. Recently, there are many novel technologies are applied for isolating rare cells based on both of physical and biological properties. Runze Jiang et al.[24-28] presented the NanoVelcro Chip platform, which coated with biotinylated anti-EpCAM on the nanofibers, combined with a laser capture microdissection (LCM) microscope to isolate the captured CTCs and fetal cells. CTCs can be captured when flow into the PDMS chaotic mixer. Shannon L. Stott et al.[29, 30] use a microfluidic device, the herringbone-chip, or "HB-CTC", to

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increase the number of interactions between CTCs and the antibody-coated chip surface. The modified device, NP-^{HB}CTC-Chip [30], which is coated a gold nanoparticle on the surface of ^{HB}CTC-Chip by chemical ligand-exchange reaction, is easy to detach the captured CTCs from the substrate for downstream application.

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1.3 Purpose of this research

The purpose of this research is to develop a microfluidic system to enrich extremely rare fetal cells in maternal circulation. These targets are enriched using a centrifugal microfluidic disk with seamless on-disk staining for fluorescence marks identification. The target cells are then retrieved by single cells pick technique to enable comprehensive downstream genetic analyze, including whole genome amplification, polymerase chain reaction and shore tandem repeat. Specifically, JEG-3 cell line was used as target cells to optimize the entire workflow. Special feature in the collection of target cells during the enrichment process was designed to ensure maximum capture of these rare target cells as well as consideration of workflow optimization to prevent cell lose. Taken together, this research should provide a microfluidic device to enable non-invasive prenatal diagnosis with intact fetal whole cells.

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Chapter 2 Design Feature and Methodology

2.1 Disk design

The principle of isolating the fetal cells in the thesis is based on density gradient separation due to the different density of various cells(Figure 1. A). The main effect of DGS is to separate the RBCs and macrophage during high-speed centrifugation. The principal advantages of density-based isolation method is mark free and enrichment of fetal cells, but it may lead to large variation in recovery rate due to different operator.

The advantage of the chip-based microfluidic device is automate sequential processes reducing the variation of operator, including density-based enrichment of fetal cells and multiple immunofluorescences staining. The relative centrifugal force with dimensionless quantity is presented as:

$$\mathrm{RCF} = \frac{r\omega^2}{g} \ , \tag{1}$$

where g is the standard acceleration due to gravity, r is the rotation radius and ω is the angular velocity in radians per unit time.

For the suspended cell centrifuged in the disk during the isolation process, it will sustain three different forces, centrifugal force F_c, buoyancy force F_b and frictional force between the cell and surrounding F_f (Figure 1. B). The net force acts on a cell can be written as:

$$\sum F = F_c + F_b + F_f = \mathrm{ma} \ , \tag{2}$$

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If the density of cell equal to the solvent that also celled DGS, the cell will not move relative to the solvent, and the velocity along the radius becomes zero. Generally, the density of erythrocytes and granulocytes are about at the range of 1.090-1.110 g/ml, lymphocytes and monocytes at 1.059-1.077 g/ml, and plasma or serum at 1.026 g/ml. Therefore, the DGS of 1.084 g/ml can be used for the enrichment of fetal cells, which density is smaller than 1.084 g/ml.

When the cell reaches the equilibrium state, which means centrifugal force is equal to buoyancy force and friction force, it will settle with terminal velocity. The equation is presented as:

$$\mathrm{mr}\omega^2 - m\nu\rho_f r\omega^2 - f\frac{1}{2}\rho_f U_s^2 \mathrm{A} = 0 \ , \qquad (3)$$

where m is mass of the cell, ν is the specific volume of the cell(volume per unit mass), ρ_f is the density of surrounding solvent, f is the drag coefficient, U_s is terminal velocity and A is reference area.

The red blood cells settle to the bottom of separation reservoir because buoyancy force and friction force are less to the centrifugal force, whereas target cells will reach the equilibrium state at the DGS-plasma interface in radius direction. The diluted blood sample and the RB+EDTA using for dilution simultaneously is continuously infused into the disk, it will drive cells to outlet of the disk. Moreover, the driving force that makes cells go into the collector chip is the relatively high water level compared to the outlet of

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chip, the equation form can be shown as:

$$q = \frac{Q}{b} = \int_{R_g}^{R} \sqrt{2\bar{r}\omega^2 r}\, dr \ , \qquad (4)$$

where q is the flow per unit width, $\bar{r}$ is the average radius of the fluid, R is the radius of the upstream fluid, and R_g is radius of the downstream fluid.

From the equation, the main flow is driven by the centrifugal force term. Besides, R, will increase as fluid is infused, causing a relatively high pressure to drive cells forward.

The top of DGS in the disk of separation interface is continuously rinsed by injected blood sample, so it will cause variation in density and target cells may be sediment in the disk unable to be collected under the retrieval position. To solve this problem, 1.5 ml DGS will be added into the disk after separation process to raise the interface and push cells into the collector.

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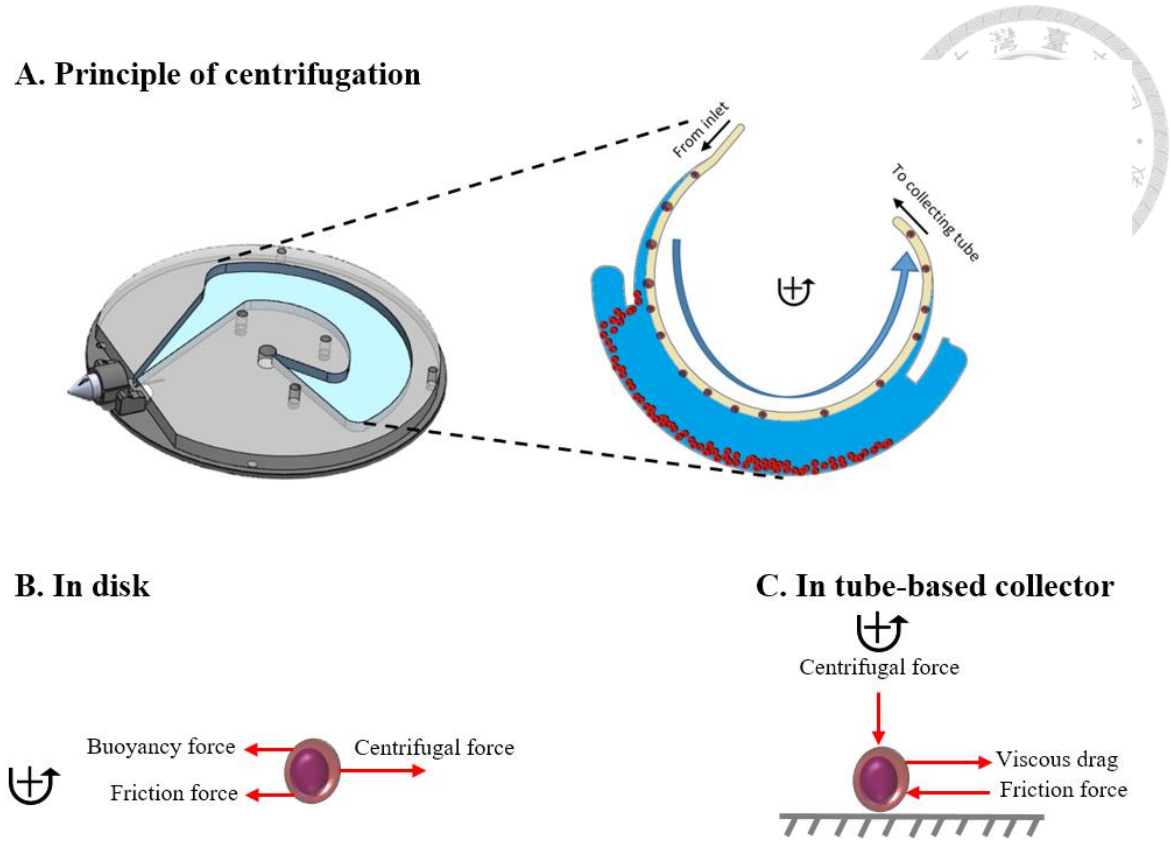

Figure 1 Forces acting on a cell. A. Suspended particle is balanced by centrifugal force F_c, buoyancy force F_b, and frictional force between the cell and surroundings F_f. B. Forces acting on a cell attahced to the bottom of tube-based collector. C. Principle of centrifugation.

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2.2 Tube-based collector design

To enable rapid verification of the system, cell models were pre-stained with nuclear dye Hoechst 33342 and HLA-G-PE. The experiment time was decreased by pre-stained the cell models and every single data point was assayed in triplicate. The recovery rate in this thesis is defined as:

$$\text{Recovery Rtae } (\%) = \frac{Number\ of\ Detected\ Cells}{Number\ of\ Spiked\ Cells} \times 100\ (\%)$$

When the target cells are isolated from the disk and going into the tube-based collector, the main affect factors become centrifugal force and lift force (Figure 1. C). Those two forces can be used to determine whether the target cells stay or flow out with the waste stream. The effect of sedimentation can be estimated by terminal sedimentation velocity U_s and is presented as:[31]

$$U_s = \frac{\rho_c - \rho_f}{18\mu} D_c^2 r_d \omega^2 \ , \qquad (5)$$

where ρ_c is density of the cell, ρ_f is density of the fluid, μ is the fluid viscosity, D_c is the cell diameter, r_d is the radius of rotation, and ω is the angular velocity of rotation.

The equation (5) shows that if density of the cell is larger than the fluid (i.e., $\rho_c > \rho_f$), the friction force generated by normal force will make the cell attach to the bottom of tube-based collector. On the other hand, if the density of the cell is smaller than the fluid (i.e., $\rho_c < \rho_f$), it may flow out with the waste stream. The drag force came from the fluid can be written as:

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$$F_g = 6\pi R\eta\vec{v} \ , \qquad (6)$$

where F_g is viscous drag force, R is spherical particle, $\vec{v}$ is moving speed, and η is viscosity of a medium.

Therefore, the density in tube-based collector should be relative lower than the isolated cells, or the isolated will be flushed away. During the isolation process, some DGS is raised by sediment cells and go into the tube-based collector, which will cause density variation. So, the RB+EDTA is used to dilute simultaneously during the separation process, which enables to keep a relatively low density and resist cells flow out of the collecting tube.

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2.3 Tube-based collector immunofluorescence staining

Cells will go into the collecting tube after separation process, staining reagent is added into the rotating disk and runs along the channel into the collecting tube. Original solution will be replaced by staining reagent. The motor will be stopped and mix well by a pipette, and then incubate for a requirement of staining time with different staining reagent. The whole staining process is shown in figure 2.

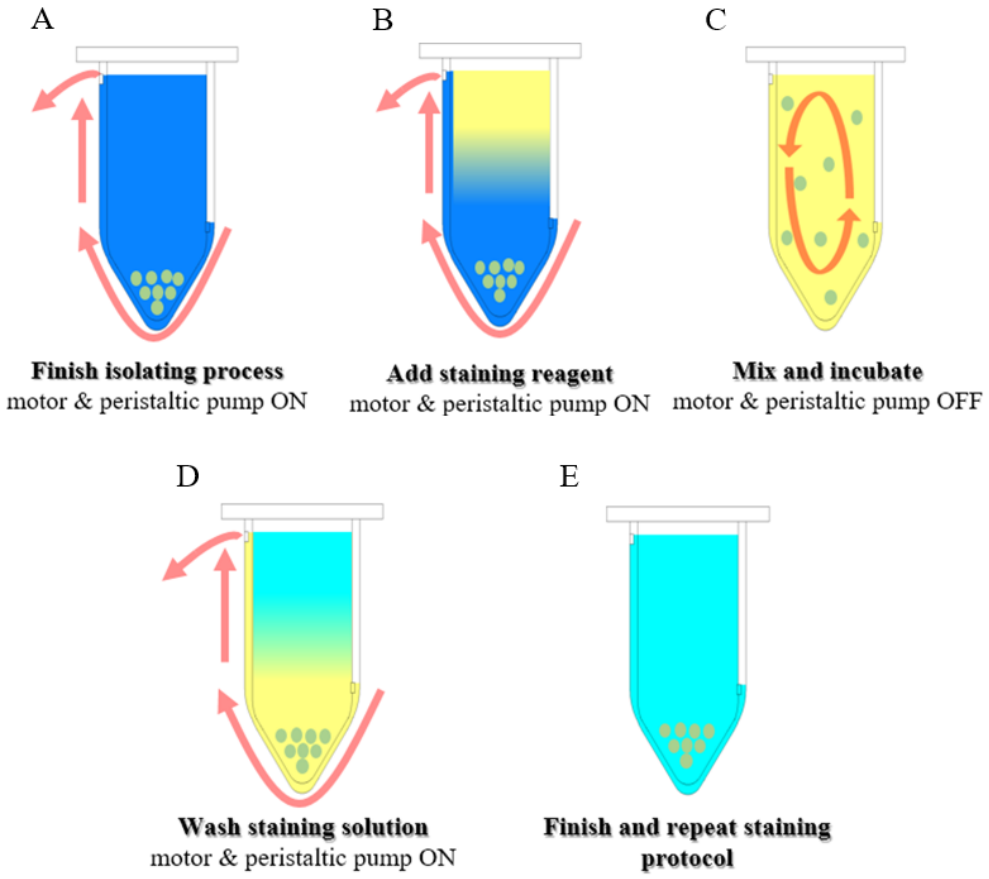

Figure 2 Immunofluorescence staining steps inside the tube-based collector. A. Finish the isolating process. B. Add staining reagent. C. Mix and incubate D. Wash staining solution. E. Finish and repeat staining protocol.

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Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Fabrication of disk and tube-based collector

The microfluidic device(Figure 3 C) was mainly divided into two parts, centrifugal microfluidic disk and tube-based collector. The centrifugal microfluidic disk was made by stacking three layers of PMMA material, a bottom layer, an isolation layer, and a top layer. The tube-based collector was composed of two layers of polypropylene (PP), inner layer and outer layer and the tube holder was made steel(Figure 3 A and B). The microfluidic device needs to be confirmed it will not have liquid leakage.

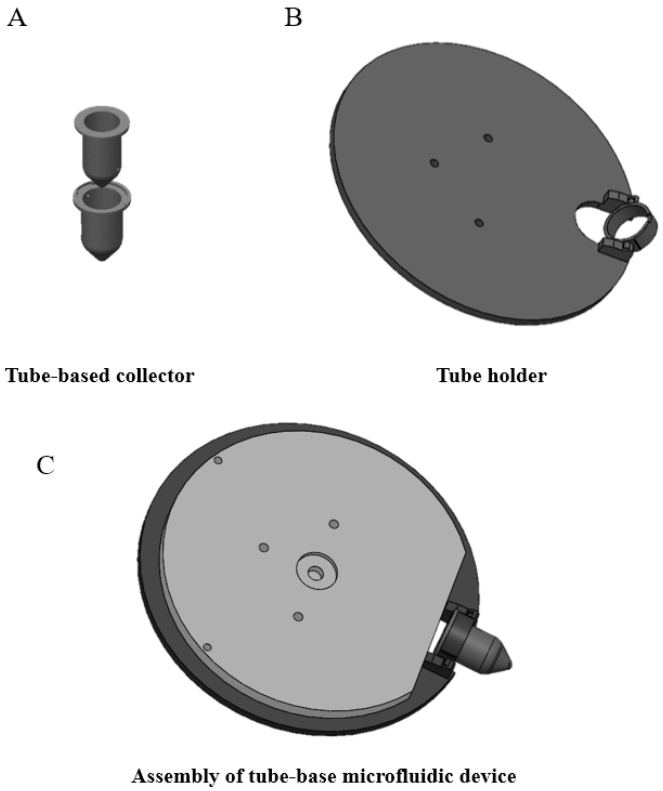

Figure 3 Fabrication of the collecting device. A. The tube-based collector was made by polypropylene. B. The holder was made by steel. C. The assembly of microfluidic device

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3.1.2 Cells and cell culture

To verify the microfluidic device is feasible and stable for isolating fetal cells from maternal blood, the JEG-3 cell line is introduced to be the cell model. JEG-3 cell line, which came from human placenta choriocarcinoma, can be also used to test the expression level of the surface markers, such as HLA-G-APC and CK-PE.

JEG-3 cell line was cultured in the Eagle's minimal essential medium (EMEM, ATCC® 30-2003™), which mix with 10% fetal bovine serum, 1% penicillin-streptomycin and 1.2g/L sodium pyruvate, and grown in Petri dishes at 37℃ under an atmosphere of 5% CO_2.

3.1.3 Reagents

There are various reagents used in this thesis such as various solutions, PCR reagents, and WGA reagents. The detail formulas are as below tables.

Table 1 Various reagents

Target/human	Vendor	Cat. No.	Clone
Fixation buffer	BioLegend	420801	-
Inside perm	Miltenyi Biotec	NC9962886	-
TransFix	Cytomark	TFB-20-1	-
Hoechst 33342	Thermo Fisher Scientific Inc.	H3570	-
CD-45, IgG	Milteny Biotech	130-080-202/mono mouse	5B1

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HLA-G, IgG		Milteny Biotech	130-111-852/mono mouse	87G
CK	C-11, IgG	Abcam	ab56420/ mono mouse	C-11
	PanCk, IgG	eBioscience Inc.	41-9003-82/ mono mouse	AE1/AE3

Table 2 The reagents for PCR kits

Reagents	Volume(μl)
Forward primer	3
Reverse primer	3
Pro Taq^{TM} plus DNA polymerase	0.5
10XTaq buffer	5
dNTP	1
H_2O	27.5

Table 3 The primer for PCR

Chosen primer	Sequence (5′－3′)
G3PDH-forward	TGACCTTTCTGTAGCTGGGG
G3PDH-reverse	CAAGCCCACCCCTTCTCTAA
SRY-forward	GAATATTCCCGCTCTCCGGAG
SRY-reverse	ACCTGTTGTCCAGTTGCACT
Ch.3-forward	TTCACCCAACATCATCCGGG
Ch.3-reverse	GCCTCTGCAATCCTTATCCTCA
Ch.11-forward	TAGTCCCACTGTGGACTACTT
Ch.11-reverse	CCTGAGAGCTTGCTAGTGATT
Ch.13-forward	AGCAGGCAACACAATGCAAG
Ch.13-reverse	CAGGCAGGGATGGGTTTGAT

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Table 4 The reagents for WGA kits

Reagent		Volume(μl)
Buffer D2	DTT, 1M	3
	Reconstituted buffer DLB	33
Master mix	H_2O sc	9
	RELI-g sc	29
	RELI-g sc DNA polymerase	2

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3.2 Methods

3.2.1 Isolation of fetal cells from whole blood via disk system

The whole workflow is shown in figure 4 after receiving a pregnant blood, then applied to the isolation device and staining. Afterward, the isolated cells are applied to secondary purification and downstream genetic application. In order to verify the microfluidic device, about 100~200 cells mentioned above were spiked into 4 ml whole blood, which came from healthy donors. First, added the 200ml of TransFix reagents into a 4ml whole blood, which spiked cell model, and incubated for 20minutines at room temperature. Afterward, the blood sample was continuously loaded into the spinning disk by peristaltic pump, RBCs were sediment at the separation reservoir and the target cells were flow into the tube-based collector.

After the isolating process, there were several immunofluorescence-staining steps going to be finished. Firstly, the collected cells incubated with 1.5 μl of Hoechst 33342, 10 μl of HLA-G-APC, 5 μl of CD-45-FITC, and fixation buffer for 25 minutes and washed with RB+EDTA. Secondly, the collected cells incubated with permeabilized buffer for 15 minutes and washed with RB+EDTA. Finally, the collected cells incubated with CK-PE for 35 minutes and washed with RB+EDTA.

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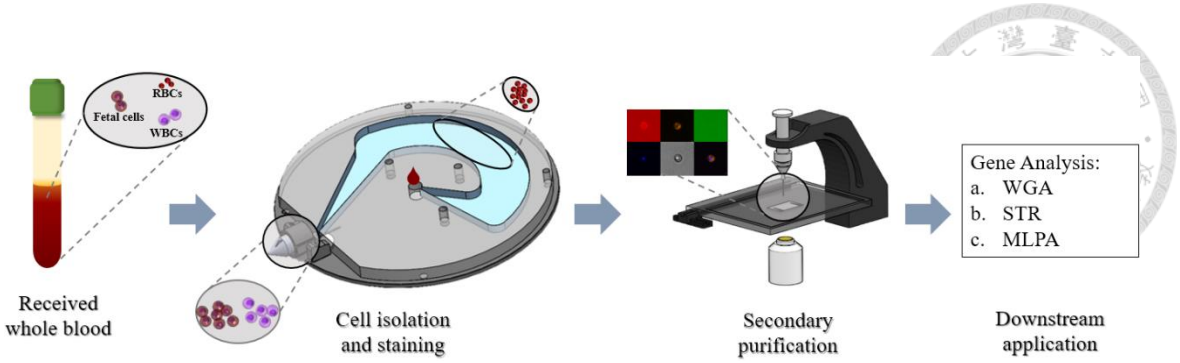

Figure 4 The whole workflow of NIPD including the enrichment of fetal cells, cell detection, secondary purification, and downstream genetic application.

3.2.2 Primary sample preparation

There several steps for primary sample preparation after receiving a placenta sample from a pregnant woman. Firstly, chopped the sample into fragment in a dish, which contained 1 ml medium, and removed tissue fragments to a 15 ml tube, then use 5 ml collagenase-containing medium (10 mg/5 ml) to move the remaining cells in the dish to the tube. Secondly, sealed the tube and shake it vigorously at 37℃ for 40 minutes, then applied centrifugation (at 4℃, 1300rpm for 3 minutes) to remove the supernatant. Thirdly, added 2 ml of 0.05% trypsin to dissociate cell aggregates for 2 minutes with sufficient pipetting, then added 2 ml of medium to reduce the availability of trypsin. Finally, applied centrifugation (at 4℃, 1300rpm for 3 minutes) to remove the trypsin-containing medium and resuspend cells in 5 ml by medium, then used a 70 μm cell strainer to sieve cell aggregates. After the above primary sample preparation steps, the placenta cells were ready to be used.

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3.2.3 Automatic scanning and secondary purification

The target cells were identified as fetal cells if they are Hoechst 33342 positive, CD-45-FITC negative, HLA-G-APC positive, CK-PE positive, and is morphologically distinct from surrounding WBCs.

3.2.4 Whole genome amplification and polymerase chain reaction

The workflow of WGA and PCR followed the protocol given by Qiagen Corporation. The parameters of PCR were as:

Table 5 The PCR condition

Steps	Temperature(℃)	Time(s)
Denaturation	95	300
	95	30
Annealing	62	30
Extension	72	60
Cycle	40	
Incubation	4	∞

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Chapter 4. Results and Discussion

4.1 Validation of characteristics of tube-based collector

Validation of the system performance of the microfluidic design involves a range of parameters. In section 4.2, the effect of Ficoll (DGS) on the recovery rate will be discussed. In section 4.3, parameters affecting yield of spiking primary cells are discussed, in which the effect of different cell models and the Transfix solution (Cytomark Company) are tested. Comparison of recovery rates between the microfluidic device and the chip-based microfluidic device are also tested in this section. Finally, section 4.4 will present results on the whole process with isolation, downstream analysis such as WGA, PCR, and STR. A positive selection method was also test and the results see the appendix.

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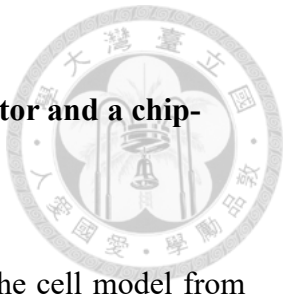

4.2 Comparison of recovery rate between the tube-based collector and a chip-based collector.

It is important to select an appropriate DGS that can separate the cell model from the pool of background cells. Toward this end, three different DGS, 1.077, 1.084 and 1.119 g/ml, were tested. About 200 JEG-3 cells were spiked into 4mL whole blood each time in these three different DGS and processed under the same DGS process by the chip-based device. Figure 5 presents the recovery rate of three different DGS, recovery rate for 1.077 g/ml is 61.7±14.1 %, recovery rate for 1.084 g/ml is 85.2±11.2%, and recovery rate for 1.119 g/ml is 49.2±6.1 %. The results show the density of 1.084 g/ml is the most efficient and its recovery reaches 85.2 %±11.2%, which indicates that the density of most JEG-3 cell line is smaller than 1.084 g/ml and larger than 1.077 g/ml. However, the reason why the recovery rate of the density of 1.119 g/ml was lower than 1.084 g/ml was because the density in chip-based collector was raised by the DGS and the sediment cells were wash out of the chip-based collector. The density of 1.119 g/ml cannot be applied to our device, therefore, this DGS will not be tested.

After finding the proper DGS, the recovery rate of microfluidic device was also investigated and compared with chip-disk device. Figure 6 presents the recovery rates between the chip-based collector and tube-based collector are 85.2 ±11.2% and 72.4±2.9% respectively. After using unpaired t test to analyze those two data, the results

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show there are no statistically significant difference between tube-based collector and chip-based collector, so the tube-based collector can replace the chip-based collector.

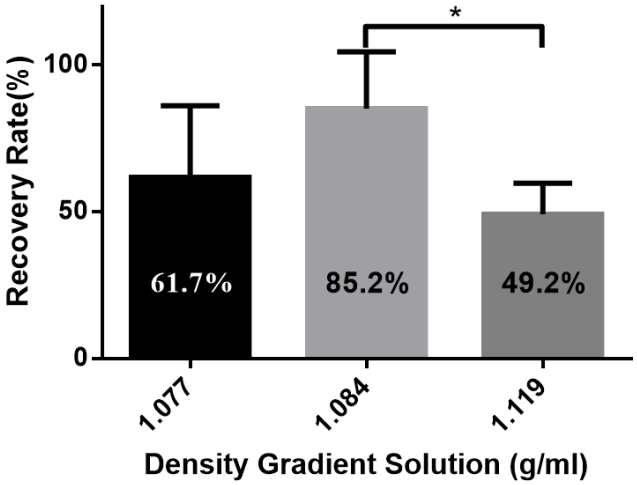

Figure 5 ***Recovery rate of JEG-3 cell line with different density of gradient solution. About 200 JEG-3 cells were spiked into 4 ml blood, and the recovery rate was 61.7±14.1 %, 85.2±11.2%, and 49.2±6.1 % for the density of 1.077, 1.084 and 1.119 g/ml. (* means p value<0.05.)***

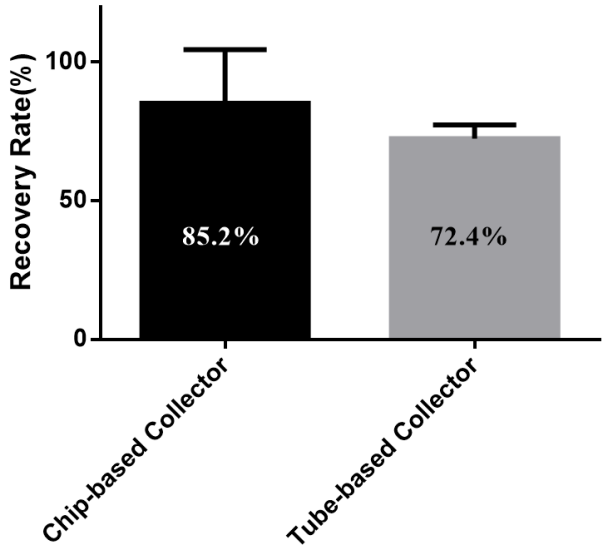

Figure 6 Comparison of recovery rates between the tube-based collector and the chip-based collector. About 200 JEG-3 cells were spiked into 4 ml whole blood from a healthy donor. Results showed a recovery rate of chip-based collector is 85.2 ±11.2% and tube-based collector is 72.4±2.9%, there is no any statistically significant difference between them.

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4.3 Characteristics of placenta cells

To further evaluate the microfluidic device, primal cells derived from maternal placenta were also studied. All the placenta cells were harvested at the same process mentioned in section 3.2.2. Placenta cells were spiked in a pure running buffer rather than whole blood and then did the DGS process with tube, to find out which DGS can isolate the placenta cells most efficiently. Figure 7 presents the recovery rates of placenta cell in three different DGS with the same isolation process by tube. The results show that DGS of 1.084 g/ml is higher than 1.077 g/ml and has statistically significant difference between them, so the 1.084 g/ml was used in the next test. More specifically, the recovery rate for 1.077 g/ml DGS 19.9±2.3%, recovery rate for 1.084 g/ml is 33.4±1.7%, and 1.119 g/ml for 48.2±3.4%. As discussed in section 4.2, the density of 1.119 g/ml cannot be applied to the chip-based microfluidic device due to a raised density in the collecting chip, so it will not be discussed in this section.

Figure 8 presents the recovery rates of placenta cell is 22.3 ± 5.2% and JEG-3 cell line is 72.4±2.9%, the recovery rate of using placenta cell is much lower than using JEG-3 cell line. The main reason for having the low recovery rate was speculated that placenta cells were too fragile to afford centrifugal force, its will break during the isolation process. In order to reduce the damage during the DGC process and prevent cellular degradation, 200μl of the TransFix reagent was added into 4 ml blood that simultaneously contains

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placenta cells and then uses the same process to enrich target cells.

Figure 9 presents the recovery rates of chip-based microfluidic device and microfluidic device whether has the TransFix reagent or not, figure 9A shows the results of chip-based microfluidic device and figure 9B shows microfluidic device. The performance, which contains TransFix reagent, is higher one either chip-based microfluidic device or microfluidic device. So, TransFix reagent will be used for the NIPD process in the future.

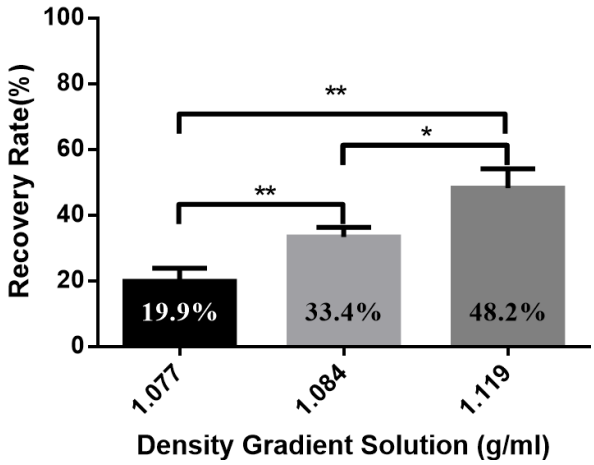

Figure 7 Recovery rates of placenta cell with different DGS. Approximately a hundred placenta cells were spiked into 4 ml running buffer, and the recovery rate was 19.9±2.3% for 1.077 g/ml, 33.4±1.7% for 1.084 g/ml, and 48.2±3.4% for 1.119 g/ml. (* means p value<0.05, ** means p value<0.01 .)

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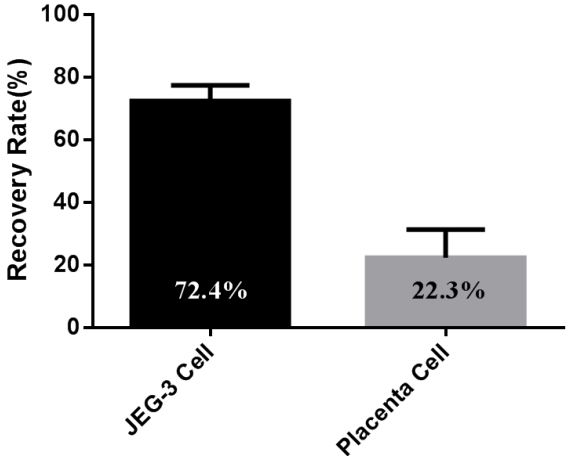

Figure 8 Comparison of recovery efficiency between placenta cell and JEG-3 cell line by using tube-based collector. About a hundred cells were spiked into 4 ml whole blood from a healthy donor. Results present a recovery rate of 72.4±2.9% for spiking JEG-3 cell line and 22.3 ± 5.2% for spiking placenta cell.

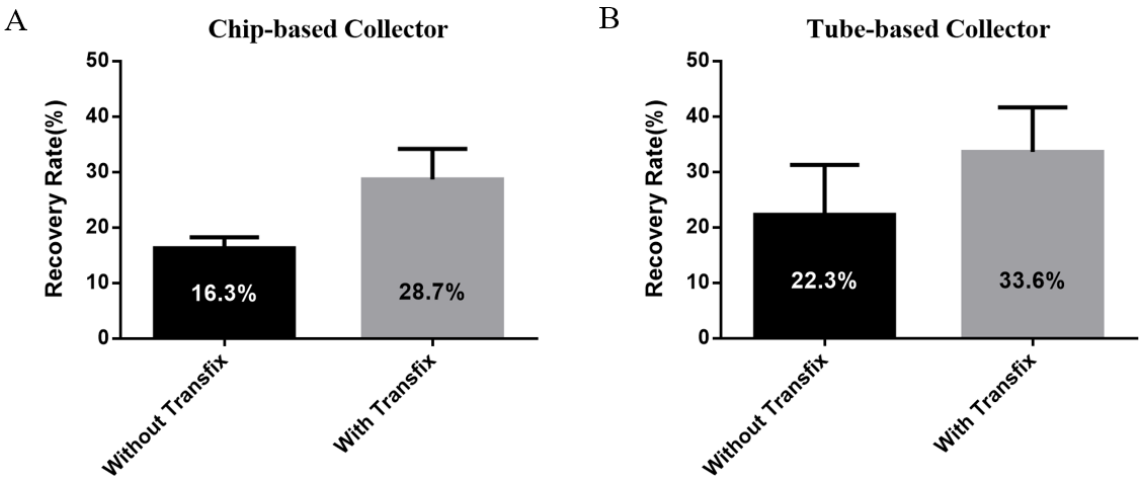

Figure 9 Verification of efficiency of the TransFix solution. About a hundred cells were spiked into 4 ml whole blood from a healthy donor. A. Recovery rate of 28.7±3.2% for chip-based collector with TransFix reagent and 16.3±1.1% for chip-based collector without TransFix reagent. B. Recovery rate of 22.3±5.2% for tube-based collector with TransFix reagent and 33.6±4.7% for tube-based collector without TransFix reagent.

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4.4 Downstream application and verification

To verify our target cell can be applied for downstream gene analysis after the isolation process, multiple different chromosomes are chosen to confirm the cells, containing the target cell's genome. JEG-3 cells and primary placenta cells, which were pre-stained with Hoechst 33342, cytokeratin, and HLA-G, were spiked into 4 ml whole blood from a healthy donor.

After using microfluidic device to isolate target cells from whole blood, the single cell picking system was used for secondary purification. Multi-chromosome kits were used to confirm the cell after secondary purification, such as SRY, G3PDH, chromosome 3, chromosome 11, and chromosome 13. Figure 10 presents the results after WGA and PCR, figure 10A shows the retrieved JEG-3 cells and figure 10B shows the placenta cells after isolation process and single cell picking steps, both of them have positive amplification.

The retrieved JEG-3 cells were also confirmed by using commercial kits to do STR and the Figure 11 is the results of profile. There are six different loci using for STR analysis, such as D19S433, vWA, TPOX, D18S51, D5S818 and FGA. The results shows that the peak of retrieved JEG-3 cells are the same as the JEG-3 cell line and different from the healthy donor.

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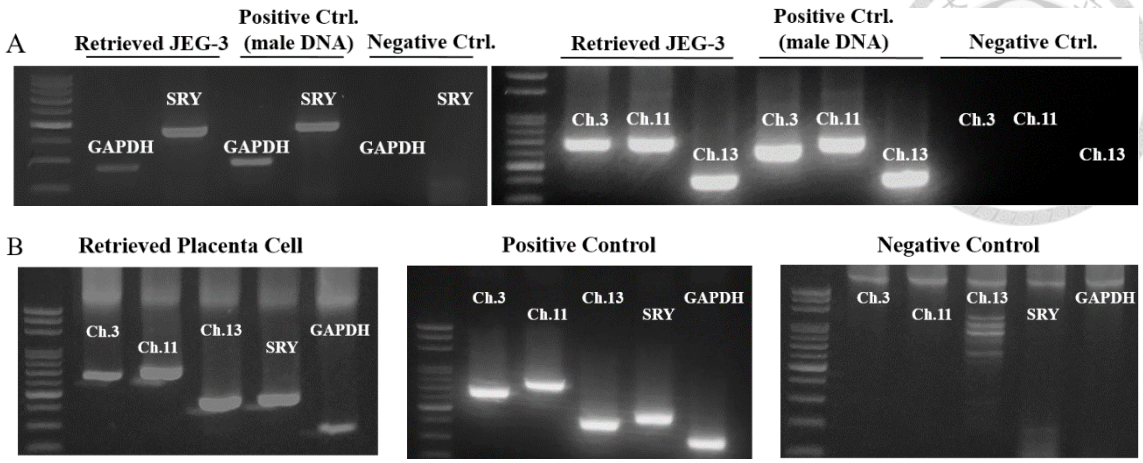

Figure 10 JEG-3 and placenta cells were pre-stained with Hoechst 33342 and HLA-G-PE, and spiked into 4 ml whole blood. After isolation process and single cell picking, the ch.3, ch.11, ch.13, SRY, and GAPDH were selected to confirm the retrieved cells by WGA and PCR. A. The results of JEG-3 cells after retrieved process and confirmed by PCR. B. The results of placenta cells after retrieved process and confirmed by PCR.

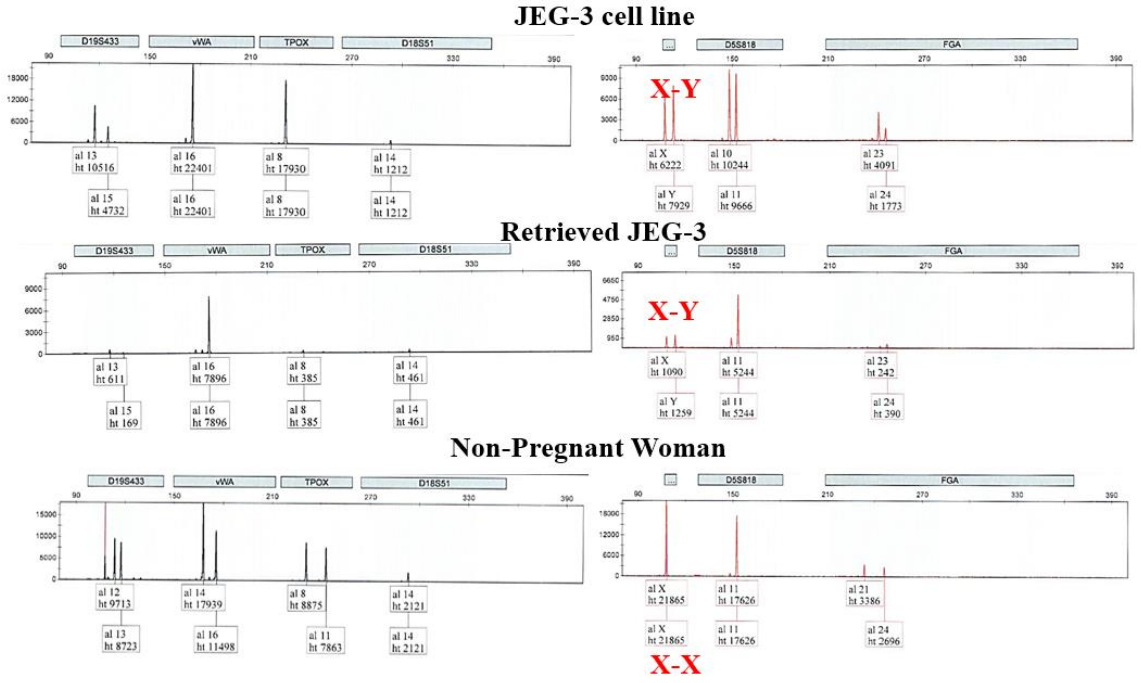

Figure 11 STR profiles of JEG-3 cells spiked in non-pregnant woman's blood after the whole process. There are six loci using for STR, such as D19S433, vWA, TPOX, D18S51, D5S818 and FGA. The results show that the alleles of retrieved JEG-3 cells were the same as the JEG-3 cell line and different from the non-pregnant woman's blood which spiking the JEG-3 cells.

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4.5 Further discussion

To verify the immunofluorescence-staining steps will not affect the recovery rates. About 200 of JEG-3 cells were spiked into 4 ml whole blood and went through the isolating process and staining step contained HLA-G-APC, CD45-FITC, and Hoechst 33342. Figure 12 presents the results of fluorescence images after staining cells including target cell and background cells. Figure 13 shows the comparison of recovery efficiency between pre-staining and post-staining JEG-3 cells. More specifically, the recovery rate of the pre-staining cell is 77.3±5.3% and post- staining cell is 79.5±3.5%.

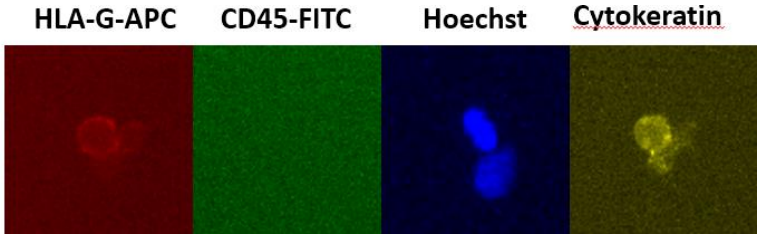

Figure 12 Fluorescence images of stained JEG-3 cell line. JEG-3 cell was confirmed as target when it has signal of HLA-G+, CD45- ,CK+, contains nuclear expression and has a clear morphology.

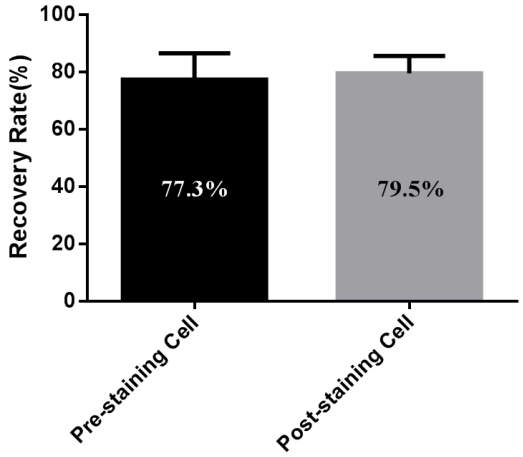

Figure 13 Comparison of recovery efficiency between pre-staining and post-staining cells. About 200 JEG-3 cells were spiked into 4 ml whole blood from a healthy donor. Results present a recovery rate of 77.3±5.3% for pre-staining JEG-3 cell line compared to 79.5±3.5% for the post-staining one.

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Chapter 5 Concluding remarks

Many research groups are devoted to isolate fetal cells from maternal blood because of the importance on clinical impact. In this thesis, a novel microfluidic device and established downstream genetic application were designed. The automation of density separation method using the microfluidic device including isolation of fetal cells from maternal blood and immunofluorescence staining was demonstrated successfully and its recovery rate can reach 79.5±3.5% from 4 ml whole blood. The primary cells were too fragile to sustain the centrifugal force during the isolation process, so TransFix reagent was introduced to make the primary cells are stronger and the recovery rate can reach 33.6±4.7%.

The collected cells were applied for downstream genetic application, including WGA, PCR, and STR, the results all had positive signal. From above, the whole workflow are set up and ready for pregnant blood came from hospital.

The microfluidic device worked well, but there is still some things can be done in the future. (1) Isolate the fetal cells from pregnant blood by tube-base microfluidic device. (2) Provide a non-invasive prenatal diagnosis for pregnant women.

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Appendix

Positive selection can further elevate the purity of target cells after isolation process and make the single cell picking step simpler. Therefore, we tested a novel positive selection method that can integrate to our device easier. Figure 14 shows the concept of positive selection by electromagnetic rod. The novel positive selection device was mainly divided into two parts, a permanent magnetic rod with diameter of 1.7mm and length is equal to 30mm, and a flow tube.

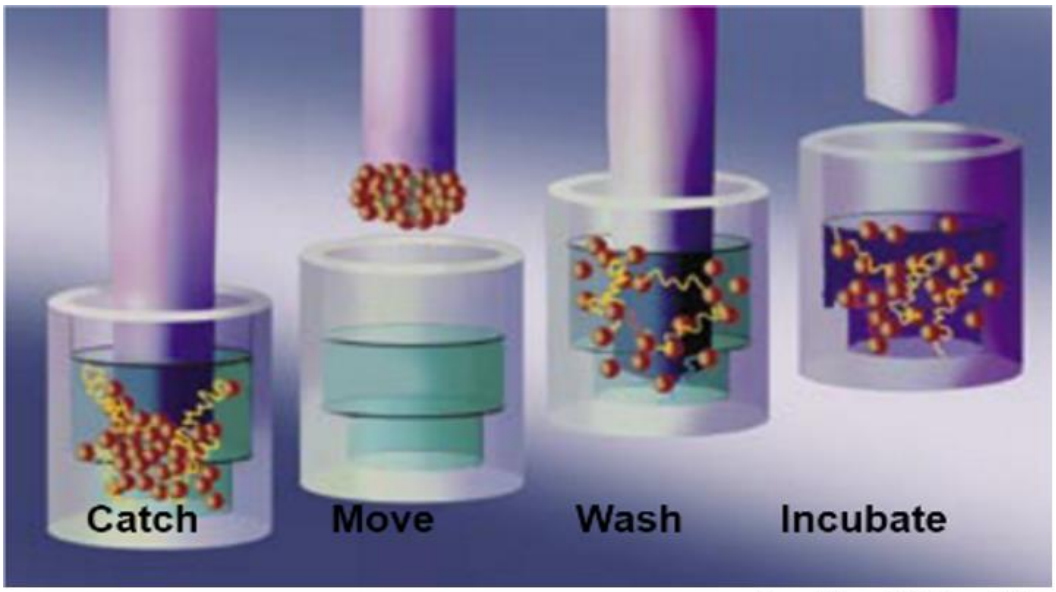

Figure 14 The concept of positive selection by electromagnetic rod. There are four steps including catch, move, wash and incubate[32].

Figure 15 presents the whole workflow of the positive selection method. Firstly, the target cells were spiked into the flow tube, which contained background cells. Secondly, after mixing, the magnetic rod was soaked into the flow tube and wait for 10 minutes. Thirdly, pulled up the magnetic rod about 2 to 3 millimeter and waited for five minutes.

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Finally, repeated the same steps until the magnetic rod out of the flow tube. Afterwards, the magnetic rod was directly soaked into the lysis buffer and raised the temperature of lysis buffer up to 65℃.

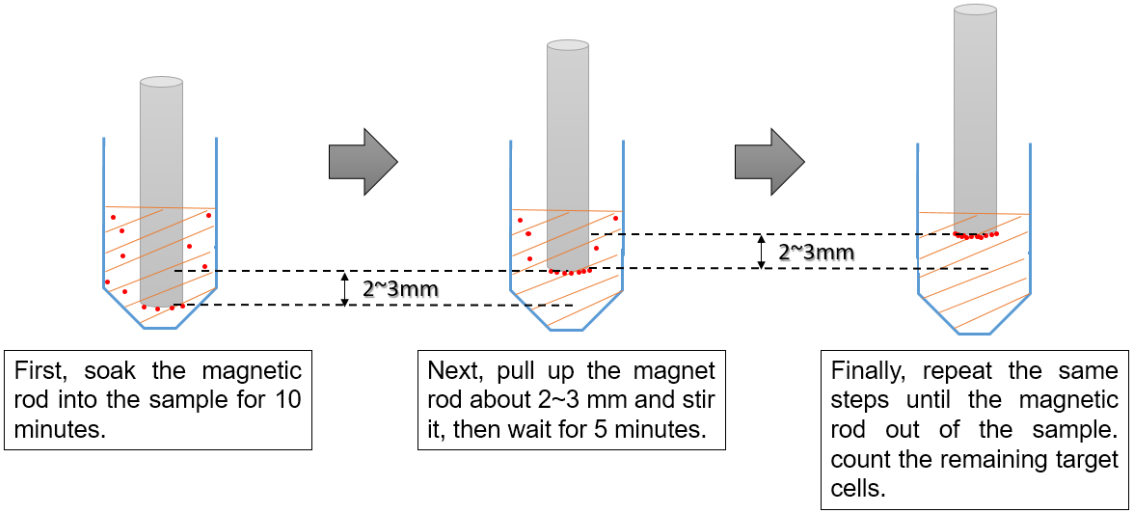

Figure 15 The whole workflow of the positive selection method.

The recovery rate here is defined as:

$$\text{Recovery Rate}(\%) = \frac{\text{Number of Spiked Cells Number of Remaining Cells in the Flow Tube}}{\text{Number of Spiked Cells}} \times 100\ (\%)$$

Table 6 Results of positive selection by magnetic rod.

Spiked cell line	Number of spiked cells	Recovery rate	Result of PCR
JEG-3	116	85.3%	Positive amplification
JEG-3	50	88%	Positive amplification

Table 6 shows the recovery rate of the positive selection by magnetic rod can reach more than 80%. Further, the PCR was also used and verified that positive amplification was achieved.

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