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論文の内容の要旨

1. Introduction Conventional prenatal fetal DNA diagnosis methods are invasive when fetal cells are recovered, and it has a constant risk of miscarriage of the fetuses. For example, amniocentesis has a risk of miscarriage and infection with 0.2~0.3 % rate. Even though, prenatal fetal diagnosis is implemented 5 million times per year in the world. It should be non-invasive to fetus. Recently, the researches for non-invasive prenatal diagnosis using maternal peripheral blood were studied. In the maternal peripheral blood, nucleated red blood cells (NRBCs) that have migrated from the fetus exist and it is possible to analyze the retained DNA in them. However, the concentration of NRBCs in the maternal blood is very rare for example, 1~2 cells/mL, so that the separation of them is very difficult. Takabayashi et.al., reported that NRBCs were recovered successfully from the maternal blood by combination of density gradient centrifugation and image processing, and have also been successful in gender diagnosis. However, the method took too long time of 20 hours to recover the NRBCs and is inefficient for practical use. To overcome this problem, further concentration of NRBCs between the density gradient centrifugation and the image processing was effective because it reduces the number of blood cells to be searched over. To do this, we focused on the size and hardness of blood cell. Filters and chromatography also can separate blood cells, but it is difficult to recover trapped blood cells. Here, we developed micro-fluidic chip with separation/concentration and recovery mechanism by applying semiconductor micro-fabrication technology. In order to shorten the time for the separation and concentration of NRBCs by chip, we tried to develop multiple micro-gap chip. In addition, we develop fully automated processing equipment aiming at improvement of reproducibility and convenience. From those, the efficiency for the recovery of NRBCs was improved very much, which contributes to realize noninvasive prenatal genetic diagnosis.

2. Experimental The design of micro-gap chip was done using Adobe Photo Illustrator. Micro-gap chip is fabricated by photolithography. The height of the gap of the micro-gap chip was made at 1.2 μm, 1.5 μm, and 1.9 μm. As preparation, maternal blood was centrifuged by density gradient. Maternal blood was delivered to the micro-gap chip, with the flow rate to the chip of 1 μL/min, and the process time for separation and concentration of 30 minutes. Red blood cell, white blood cell count was measured using FACS. In order to speed up nucleated red blood cell separation by chip, multiple micro-gap chip was designed with

Adobe Photo Illustrator and fabricated using photolithography. The number of micro-gap channels was increased 5 times from 90 to 448. Umbilical cord blood diluted 10 times was delivered to the chip. Here the flow rate to the chip was 11 µL/min, 22 µL/min, 33 µL/min. PLC (programmable logic controller) was used for automation of multiple micro-gap chip, and flow operation and diaphragm operation were carried out by air pressure control using an actuator and an air cylinder.

3. Results and discussion The blood cell can be trapped at the interstitial spaces of the micro-gap chip, and the trapped blood cells can be recovered by opening the membrane gap. NRBCs were discovered from the recovered solution. When the height of the micro-gap channel was 1.0 µm, the collection rate of NRBCs was 92%. When the micro-gap channel height was 1.5 µm and 1.9 µm, the collection rate of NRBCs was 75%, 25%. Removal rates of red blood cells and white blood cells were 93% and 98%. The processing time for one specimen was 90 minutes. The collection rate of NRBCs was 84% at the flow rate of 11 µL / min in multiple micro-gap chip. The collection rate was 66% when the flow rate was 22 µL / min and 60% when the flow rate was 33 µL / min. Removal rates of white blood cells and red blood cells were 98% and 84% at the flow rate of 11 µL/min. Those were 93% and 99% when the flow rate was 22 µL/min, and 92% and 94% when the flow rate was 33 µL/min, respectively. From these results, the time for automatic image processing can be expected to be shortened from 20 hours to 1 hour. Moreover, the number of tests can be increased from 1 sample per day to 20 samples per day. By pressure control using PLC, flow operation and membrane control were automated. The automation of multiple micro-gap chip was achieved from sample introduction to recovery.

4. Conclusion The fabricated micro-gap chip was able to trapped NRBCs. When the height of the micro-gap channel was 1.0 µm and the flow rate was 1.0 µL/min, the collection rate of NRBCs was 92%. Removal rates of red blood cells and white blood cells were 93% and 98%, respectively. In the multiple micro-gap chip, the collection rate of NRBCs was 84% at flow rate of 11 µL/min. Here, the removal rates of red blood cells and white blood cells were 84% and 98%. Using this chip, the total throughput of the recovery of NRBCs is expected to be improved by factor 20. The automation of micro gap chip operation is also achieved for convenience. From these, it is expected that the developed micro-gap chip will greatly contribute to non-invasive prenatal diagnosis.

Keyword: Noninvasive diagnosis, NRBCs, Cell hard and size, Micro- fluidic-chip, Micro gap.

論文審査の結果の要旨

本論文は、無侵襲の胎児 DNA 診断を実現するために母親の血液中に存在する胎児由来の有核赤血球細胞(Nucleated Red Blood Cell: NRBC)を微小な間隙を用いて濃縮・回収する手法の開発に関するものである。これまで胎児 DNA 診断は羊水穿刺や絨毛採取により行われていたが母体・胎児に対して一定のリスクをともなった。母体末梢血に含まれる胎児由来の有核赤血球 NRBC を分析することにより、リスクのない胎児 DNA 診断が期待できる。母体血中の NRBC は非常に希少かつ他の細胞との判別が難しいため、密度勾配遠心分離などの方法により他の細胞を除き NRBC を濃縮、その染色標本を作製し自動画像処理装置により NRBC を探索、最終的には目視で判定することで、正確な回収と高い正診率を得ている。しかし、血球の撮像と画像処理

に非常に時間がかかりその短縮が課題であった。そこで本論文では、密度勾配遠心分離後のサンプルをさらに濃縮するために、NRBC の血球の大きさと変形のし難さに注目し、チップ上に形成した拡張可能な微小間隙を用いて、NRBC の選択的な捕捉・回収を試みた。

第 1 章では、母体血を用いた胎児 DNA 診断ついてその必要性と問題点、これまでの研究についてまとめ、本論文の課題を明確にした。

第 2 章では、モデル血液としてニワトリの血液を用い、Polydimethylsiloxane (PDMS)樹脂を用いて作成した流路中の微小間隙により NRBC が捕捉できるかどうかを確認し、また間隙サイズ、流速、流量などのおおまかな条件を調べ、参考データとした。また血球の捕捉状況を調べ、チップ構造の修正を行った。

第 3 章では、片壁がダイアフラムになっている微小間隙を作成し、NRBC 捕捉後ダイアフラムを操作して間隙を拡大し捕捉血球を回収可能にした。間隙が 1.0μm の時、ヒト NRBC は効率的に捕捉され、赤血球だけでなく、白血球も通過することを見出した。実際にヒト母体血を用い、密度勾配遠心分離後のサンプルをチップに導入し、NRBC の濃縮実験を行ったところ、NRBC はは間隙 93%回収でき、93%の赤血球と 98%の白血球を除去でき、必要十分な性能が得られることを示した。

第 4 章では、実際のサンプル量を扱える実証チップを開発した。チップへのサンプル導入法を工夫することで、微量なサンプルを無駄なく処理可能とした。また微小間隙を 448 本束ねることで、処理速度を向上させた。また壊れるなどしてチップ内で失われる血球量を評価した。1 検体を 16 分で処理できた。

第 5 章では、チップの制御を自動化できる装置を開発した。デスクトップサイズの装置で必要な圧力の生成、ダイアフラムの操作、サンプルの導入と回収が自動化でき、本法が十分実用的であることを示した。

以上、本論文は、血球の大きさと変形性により有核赤血球が微小間隙で効果的に選択捕捉、回収可能なことを示し、胎児 DNA 診断のための新しい分離・濃縮技術を確立したものであり、学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。