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Title Isolation of Circulating Fetal Trophoblasts using Inertial Microfluidics for Non-Invasive Prenatal Testing

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Abstract

While non-invasive prenatal testing based on cell-free fetal DNA (cffDNA) has recently revolutionized the field of aneuploidy screening in pregnancy, it remains limited to aneuploidy and microdeletion screening and is unable to reliably detect single gene disorders. A number of recent studies have demonstrated the potential of circulating trophoblastic cells in providing cell-based non-invasive diagnosis with sequencing or array-based assays. However, considering the extreme rarity of these cells in blood, efficient, high-throughput and clinically applicable enrichment technologies are yet to be developed.

This study demonstrates for the first time the utility of inertial microfluidics for efficient isolation of trophoblastic cells from maternal peripheral blood. Under optimal operating conditions, high-recovery yields (79%) were obtained using a trophoblastic cell-line, which was subsequently confirmed with analysis of maternal blood. Feasibility of obtaining a diagnosis from cells isolated from a maternal sample was demonstrated in a case of confirmed fetal Trisomy 21 in which six fetal cells were found in a 7mL blood sample using fluorescence *in situ* hybridization. Finally, we demonstrate that trophoblastic cells isolated using inertial microfluidics could be picked and subjected to a clinically validated sequencing assay, paving the way for further validation of this technology and larger clinical studies.

1. Introduction

Invasive prenatal testing using techniques such as chorionic villus sampling (CVS) and amniocentesis aims to confirm a diagnosis of chromosomal abnormality in high-risk pregnancies as established by traditional combined first trimester screening with nuchal translucency (NT) ultrasound and measurement of maternal serum analytes (free beta-hCG and PAPP-A). The inherent risk of miscarriage involved with invasive techniques and the high false positive rate of traditional first trimester screening has been a significant barrier to widespread application of prenatal aneuploidy screening.[1] The widespread uptake of non-invasive prenatal testing (NIPT) using cell free fetal DNA (cffDNA) can be largely attributed to the vastly improved screening parameters with a higher detection rate and a substantially lower false positive rate than previous first trimester screening methods. The use of non-invasive prenatal testing techniques has substantially reduced the number of invasive procedures and thus the inherent risks of population-level screening, including patients with low-risk pregnancies.

The discovery of circulating cell-free fetal DNA (cffDNA) in 1997[2] offered access to the fetal genome without the risks of invasive testing, and the improved throughput of sequencing technologies using next generation sequencing (NGS) has established the clinical validity of

non-invasive testing for aneuploidy screening and a limited range of monogenic disorders. Currently, there are a number of commercial NIPT technologies clinically available to screen for common fetal aneuploidies (e.g. Illumina Verifi™ and Harmony™) through the analysis of cffDNA in maternal blood.[3]Although cffDNA NIPT has revolutionized aneuploidy screening in obstetric practice, there are a number of inherent limitations of the technique when considering the full range of genetic disorders encountered in prenatal diagnosis. First, although recent studies have shown that the whole fetal genome is present in the maternal circulation, distinguishing between the fetal genome and the background maternal genome is not possible, and only limited coverage of the fetal genome can be achieved reliably using current protocols. NIPT allows for the reliable detection of the common aneuploidies at high sensitivity (≥99% for trisomy 21, ≥92% for trisomy 18, and ≥87% for trisomy 13) and specificity (≥99% for trisomy 21, 18, and 13), even for low risk pregnancies.[4] Broader applications of the technique for common microdeletions, genome wide screening, copy number variants[5], single gene disorders[6] and X linked disorders have recently been developed but these screening tests are characterized by significantly lower positive predictive values. Thus, expansion of the NIPT testing portfolio to a wider array of conditions recreates the initial problematic conditions of traditional first trimester screening in which patients were subjected to invasive testing at much higher rates.

Circulating nucleic acids can be analyzed at higher genomic coverage but this is to likely remain prohibitively expensive, does not overcome the problem of maternal cell-free DNA background contamination[7] and requires complicated bioinformatics.[8] For example cffDNA from a pregnant woman could be sequenced to a depth of 195× which provided a high positive predictive value and could detect fetal *de novo* mutations (including BRAF which encodes a proto-oncogene). The cost of sequencing at such a high depth is prohibitively expensive for clinical translation even with the continuously decreasing sequencing cost.[9] It is noteworthy

that, at this stage, and in the foreseeable future, cffDNA based NIPT is likely to remain only a screening technology and clinical decision-making should be carried out only after confirmation of the findings by diagnosis from fetal cells obtained by invasive testing. It is also important to note that since NIPT has been introduced, it has drastically decreased the number of invasive procedures. As a result, due to the current limited diagnostic capacity of cffDNA this has led to a decreased diagnostic yield and hence delayed diagnosis in about 1 in 350 cases. [10]

Access to a whole uncontaminated fetal genome from a non-invasive blood sample would undoubtedly vastly improve the performance of NIPT. The fact that fetal cells are shed from the developing placenta and circulate in maternal blood has been known for over a century.[11] To this end, the isolation of intact fetal cells from the maternal circulation is potentially advantageous over cffDNA approaches as it likely will allow for more comprehensive genetic analysis in a more cost-effective manner and could potentially eliminate the need for invasive approaches. However, until recently NIPT based on circulating fetal cells has been a tantalizing yet frustrating goal due to their extremely low number in maternal blood. Extremely efficient enrichment technologies are required to isolate intact fetal cells from maternal blood with the level of purity required for reliably conducting diagnostic assays. Fetal cell isolation methods should be rapid, accurate and inexpensive in order to be truly useful in a clinical setting.[12] Techniques include density gradient centrifugation, fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS)[13] and filtration, which all have been used with moderate success to isolate fetal cells in the laboratory environment.[13] However, these technologies are typically time- and resource-intensive and have significant drawbacks including low isolation efficiencies and low specificities. Consequently, they have failed to provide a valid alternative to the current invasive antenatal testing approaches in the clinical environment.

A small number of successful cases of fetal cell isolation and subsequent genetic analysis with detection of genetic abnormalities including aneuploidy, microduplication and structural rearrangements have been recently reported and brought a renewed vigor to the fetal cell-based NIPT field.[14, 15] In most recent studies after an initial enrichment step, the enriched cell fraction is spread on a slide and automated microscopy coupled with laser capture microdissection or micropipette aspiration is used to obtain single fetal cell. The performance of the initial enrichment step is critical as high levels of fetal cell recovery/WBC depletion would allow for better clinical scalability and therefore relevance.[16] The reliance on laser capture microdissection to obtain the fetal cells adds significant complexity, cost and scalability issues to such processes thus hindering the translation into clinics. There is therefore, a significant need to develop technologies able to isolate circulating fetal cells from biological fluids in a high throughput fashion and to deliver these cells in a format readily compatible with modern genomic approaches. With these requirements in mind, we endeavored to demonstrate the feasibility of isolating circulating trophoblastic cells using inertial microfluidics.

Circulating extravillous trophoblasts are fetal cells of placental origin which occur early in pregnancy (from 5 weeks gestation) and with dimensions typically larger than white blood cells (WBCs) (>15 µm).[17]The number of circulating trophoblasts in maternal blood is estimated to be ~1-5 trophoblasts[15] per mL of blood, although this is believed to vary with gestation[17], sex[18] as well as with pregnancy conditions[19] and genetic abnormalities.[20] Importantly, extravillous trophoblasts are not found post-termination/birth.[17] Syncytial nuclear aggregates (SNA) have also been consistently observed in maternal blood. SNAs are large fragments of the outer layer of the placenta, the syncytiotrophoblast, which is formed by the fusion of progenitor cytotrophoblasts into a continuous cell layer. Increased SNA number is observed in pregnancy complications including preeclampsia[21], reduced fetal movement, intrauterine

growth restriction[22] and stillbirth when compared to healthy pregnancies.[23] The large size of trophoblastic cells and SNAs provides a mechanism for isolation which has previously been exploited by the Isolation by Size of Epithelial Tumor/Trophoblast cells (ISET) test, a filtration system developed by RARECELLS for maternal blood. This test provided 100% diagnostic sensitivity and specificity in 63 pregnancies between 9 and 11 weeks of gestation at risk for cystic fibrosis or spinal muscular atrophy.[17] However, the ISET is very labor intensive as it relies on laser capture microdissection of the isolated fetal cells among a background of WBCs, and despite these promising results, has not been translated clinically.

Recent technological advances in rare cell isolation from peripheral blood have been driven by the interest in isolating circulating tumor cells (CTCs) as a source of non-invasive molecular information about solid malignancies.[24] Cell separation technologies based on the concept of inertial microfluidics have been one of the very few approaches successfully translated clinically for the enrichment of CTCs based on size and deformation.[25, 26] CTCs are indeed generally larger than WBCs, a feature that is exploited in inertial microfluidics to achieve efficient yet high-throughput separation.[27-29]

The slanted inertial microfluidic devices used in this study have been modified from the previously reported slanted inertial microfluidic CTC enrichment device.[27, 28] A key characteristic of the slanted design is the ability to efficiently process large volumes of blood (>20ml), which is especially important in the context of circulating fetal cells as their number in a normal pregnancy is extraordinary small. **Figure 1** is a schematic representation of the experimental approach used in this study. Under optimal operating conditions, trophoblastic cells could be enriched from blood within minutes and with high efficiency, delivering the enriched population in the form of a cell suspension with small (<0.5%) WBC contamination. The enriched cell fractions can be easily analyzed using a range of downstream methods. In

this study, we demonstrate successful diagnosis of fetal trisomy 21 using fluorescence *in situ* hybridization (FISH) with automated laser scanning cytometry. Finally, we demonstrate the feasibility of performing testing using a clinically validated sequencing approach from single trophoblastic cells isolated using inertial microfluidics.

2. Results

2.1 Feasibility Study with a Trophoblastic Cell Line

In order to demonstrate the feasibility of using inertial microfluidic technology for the enrichment of circulating trophoblastic cells and optimize the operating parameters, a trophoblastic cell line was used (JEG3 choriocarcinoma cell line). Fluorescently labeled trophoblastic cells were spiked into blood collected from healthy donors which was subsequently lysed and processed through the inertial microfluidic device. Under optimal operating conditions, a recovery rate of 79% (n=6) was determined using imaging flow cytometry **(Figure 2)**. In this instance, 1000 JEG3 cells were spiked per mL of blood. Although this number of cells exceeds the expected circulating fetal cell load in helathy maternal blood, it provides better accuracy of the enrichement yield as manipulating ultra-small cell numbers is challenging and inherently associated with fairly large deviation. However, in order to better simulate a real sample, we have subsequently investigated spiking ~30 JEG cells per mL of blood in a total of 4 mL (n=4). We found that with this low number we maintained a high recovery determined with microscopy of approximately 75%. The larger breast adenocarcinoma cell line MCF-7 was also tested and a recovery of 91% was obtained (data not shown), in agreement with previous CTC studies.[28, 29]

Detailed analyses indicated that the lower recovery obtained for the JEG3 cells (vs MCF-7) is primarily due to the significant cell size variation present in this cell line. Indeed, imaging flow cytometry was used to quantify the dimensions of the JEG3 cells present in both enriched and WBC/waste outlets and as presented in **Figure 3**, the area (Figure 3A), height, length and width

(Figure 3B) of the JEG3 cells found in the waste outlet were lower than those measured for cells in the enriched outlet**.** Although only limited information is available about the dimensional characteristics of circulating fetal trophoblastic cells present in the maternal circulation, the size heterogeneity of JEG3 cells makes them a suitable surrogate which provides a realistic mimic of the recovery of heterogeneous trophoblastic cells from maternal blood.

Along with the recovery yield, the depletion of blood cells is the other essential performance indicator. WBC depletion rates of 99.5% were consistently obtained using different donors and operators. In a normal hematocrit, such a depletion rate will result in approximately 10,000 WBC/mL of blood present in the enriched outlet. Besides the performance of the device, the processing time is also an important consideration, especially in the context of fetal trophoblastic cells which are likely apoptotic and have been reported to be very fragile. Under the optimal operating conditions, the equivalent of a 10mL blood tube could be processed within 45 min (including RBC lysis, centrifugation and running of the inertial microfluidic device).

2.2 Pilot Study with a Trisomy 21 Pregnancy

After having optimized the inertial microfluidic devices using a trophoblastic cell line, we aimed to demonstrate the feasibility of obtaining a prenatal diagnosis through cytological testing of circulating trophoblastic cells isolated using inertial microfluidics. For this purpose, a blood sample from a woman carrying a fetus at high risk for Trisomy 21 was obtained from the Maternal Fetal Medicine unit at the Women’s and Children’s Hospital (Adelaide, South Australia). The blood sample was obtained at 11 weeks gestation and the fetus was subsequently confirmed as being affected with Trisomy 21 using amniocentesis and karyotyping.

To demonstrate the presence of circulating fetal cells in the sample, this maternal blood was first processed with the inertial microfluidic device, and the enriched cellular population stained with cytokeratin, CD45 and DAPI and analyzed with imaging flow cytometry. Cytokeratins, especially Cytokeratin 7 and 8, are known to be expressed in placental cells and, although it is not specific, are the most widely used markers for circulating trophoblastic cells.[30, 31] These analyses revealed the presence of 4 cytokeratin positive cellular events (from 7 mL whole blood) **(Figure 4)**. We also observed the presence of white blood cells in at least one of these clusters (cluster 3 in Figure 4) which could be an artefact induced during the sample processing or a specific interaction between the circulating trophoblastic cells and WBC in the peripheral circulation. Cytokeratin has been shown to be expressed in 76% of extravillous trophoblasts (isolated by MACS specific for CD105) in maternal circulation[32] so it is possible that more fetal cells were present in this sample. No cytokeratin positive cells/clusters were found in the blood of healthy donors used as a control. Morphological analysis of cytokeratin positive events showed these cells were clustered and/or multinucleated and thus suggestive of SNAs (Figure 4). On average, positive events had an area of 321 μm^2(min 168, max 468), an average length of 27.2 µm (min 16.5, max 37.5) and width of 18.7 µm (min 14, max 26)(**Table 1**). It has been reported that syncytiotrophoblast fragments from uterine venous blood vary from 20-200 µm and contain between 2-100 nuclei which are normally densely packed, pyknotic with abundant heterochromatin and are often tear shaped, while cytotrophoblasts are often found in cytokeratin positive clusters.[33] Moreover, trophoblasts in peripheral blood have similar characteristics and syncytial fragments normally range between 100-200 µm with approximately 30 nuclei.[34] Other characteristics of trophoblasts include multinucleated (12-14 µm), diploid (7 µm) or nucleated cell remnants (5 µm).[34] Cytotrophoblasts have been reported with diameters varying 11-14 µm and 19-25 µm.[35] In this study the inertial microfluidic device used is designed to recover cellular

structures greater than approximately 15 μm and therefore, we expect that clustered and/or large multinucleated cells would be separated in a similar manner as large mononucleated cytotrophoblast cells. Smaller trophoblasts including nucleated cell remnants might therefore not be efficiently enriched.

Next, we demonstrated the feasibility of using chromosomal FISH combined with laser scanning cytometry to obtain a diagnosis from the isolated circulating trophoblastic cells. Fetal cells were considered positive when the nuclei had two green signals for Chromosome 13 and three red signals for Chromosome 21. Under the gating conditions, events were excluded if two or more nuclei were in contact (i.e. two nuclei touching was excluded from analysis as this will display as one event with >4 signals Chromosome 21). Therefore, only single cytotrophoblast cells (not multinucleated SNAs) could be detected with this semi-automated algorithm. From the 7ml blood sample that was tested with FISH, 6 cells with three red signals for 21 chromosome were detected (**Figure 5**). This result is in agreement with the expected occurrence of such circulating fetal trophoblastic cells in maternal blood and confirmed the feasibility of obtaining a diagnostic result using the combination of inertial microfluidics and FISH. Note, however, when automatically scanning FISH slides it can be difficult to maintain plane of focus and defective hybridzation can occur resulting in missing true positive cells.[20] It has also been shown that as fetal cells are very fragile, 15% are lost during the FISH process, and 9% could not undergo FISH.[31] It is likely that the approach used here underestimates the number of circulating trophoblastic cells present in the tested blood sample.

2.3. Feasibility Study of Performing Single Fetal Cell Sequencing

In order to determine the feasibility of inertial microfluidics as an initial enrichment step before genomic studies JEG3 cells were spiked into whole blood as described previously. JEG3 cells were retrieved from the enriched fraction and single cells were amplified, sequenced, aligned to the human genome and chromosome copy number determined. This study demonstrated

successful isolation of single JEG3 cells which were able to be sequenced and chromosome copy number determined using Veriseq and MiSeq technology (Illumina) **Figure 6** presents typical profiles of a normal male cell from a healthy donor (Figure 6A) and a trophoblastic hypertriploid JEG3 cell (Figure 6B).

3. Discussion

A number of recent studies have shown the feasibility of performing diagnostic genomic testing on fetal cells isolated from maternal blood.[14, 15, 30, 36] Considering the extremely low number of these cells in the blood circulation, a prerequisite requirement is the application of an ultra-efficient enrichment technology. Enrichment facilitates and fastens the detection, and therefore the collection of the placental cells of fetal origin among the background maternal WBCs. It also reduces the occurrence of false positives. In most contemporary approaches, the enriched sample is placed on slides and scanned for the target cells which are then subsequently collected, for example using laser capture microdissection or micropipette aspiration.

Magnetic activated cell sorting (MACS) is commonly used to enrich target cells based on either specific surface expression[14] (CD141 and CD105 are common[18, 31, 32]) on the fetal cell (positive enrichment) or specific surface expression on the WBCs (negative enrichment). Under optimal conditions, MACS provides good WBC depletion (~120,000 residual WBCs from 30 ml of blood).[14] Arcedi BioTech has developed a panel of specific fetal antibodies for fetal trophoblastic cell enrichment with MACS and has been shown to recover on average 12.8 fetal cells per 30 mL. These cells could be subsequently successfully used for whole genome amplification and aCGH.[15] However, blood processing (30 mL) with MACS separation subsequent staining and spreading takes up to 7 hours and the total process blood sampling to fetal cell picking takes up to 15 hours.[14]

Density gradient centrifugation is also commonly used in the enrichment of fetal cells. However, it does not provide a high enrichment, can damage cells and is associated with

significant cell loss.[12, 37] In one instance it was reported that density gradient centrifugation resulted in the loss of 60-80% of the fetal cells and that the procedure resulted in cellular degradation[37], therefore, it was recommended to improve upon this, that aggressive manipulations should be eliminated and different gradients (other than Histopaque 1.119g/ml) and densities should be tested to minimize cell loss. Combining density gradient with a negative enrichment technique such as RosetteSep™ with antibodies such as anti-CD45 and/or anti-CD36 to crosslink and cause the sedimentation of WBC can vastly decrease maternal cell contamination.[30] The enriched trophoblasts from 30 mL of blood could then be recovered and retrieved with a semi-automated single cell retrieval device and could be subjected to whole genome amplification and a-CGH or next-generation sequencing (NGS). However, the use of this enrichment was shown to decrease the number of trophoblastic cells recovered from 0.74 to 0.36 per ml of blood[30] and CD45 depletion has also been responsible for complete loss of fetal cells.[38]

We demonstrate for the first time the use of inertial microfluidics for the enrichment of circulating fetal trophoblastic cells from the maternal circulation. The performance and clinical relevance of inertial microfluidics for the isolation of CTCs is now well established.[28, 29] A commercial instrument, the ClearCell® FX1 system from Clearbridge BioMedics, has already received CE marking. The high throughput, nature of inertial microfluidics, makes it ideal to analyze large volumes of blood, separating physically larger cells i.e. fetal trophoblastic cells from smaller surrounding blood cells. A high recovery yield (>75%) was obtained using the JEG3 trophoblastic cell line as a model, even for very low cell numbers (30 cells /mL) used to better mimic the clinical situation. Inertial microfluidics allows for fast enrichment, with the time from obtaining the blood to an enriched sample being acquired being less than 45min for 20ml of blood. Because of the known fragility of circulating trophoblastic cells, it is preferable to either process samples quickly or fix them prior to the enrichment step.[31] Fixation before

pre-enrichment with MACS can be applied, however, this resulted in clogging of the columns in 13% of cases.[31] Beside its relatively low processing time, a significant advantage of inertial microfluidics is that it is very gentle on the target cells, as shown with CTCs.[26]

We also demonstrated the feasibility of establishing a diagnostic technique using circulating trophoblastic cells isolated using inertial microfluidics. Six circulating fetal cells could be enriched from a 7 mL blood sample obtained from a Trisomy-21 affected pregnancy. It is noteworthy that the presence of genetic abnormalities (particularly Trisomy 21) has been previously reported to be associated with an increase in the number of trophoblastic cells.[20, 23] Early pioneering work using the ISET filtration system illustrated the potential of size-based enrichment of circulating trophoblastic cells. ISET could indeed be applied to detect spinal muscular atrophy in a number of fetuses.[17] Despite early promising results, the technical limitations of this system, including the reliance on laser capture microdissection which can be cumbersome and time consuming requiring an experienced user[12], has to date prevented its clinical implementation. A new approach has been recently reported based on the Nano-Velcro microchips initially developed for the capture of CTCs.[18] After an initial pre-enrichment step with density gradient centrifugation, the sample is injected in a microfluidic device presenting a PLGA roughened nanosubstrate functionalized with an anti-epithelial cell adhesion molecule (EpCAM). However, the technique takes over 5 hours to enrich the fetal cells and 2 hours to isolate cells within the chip itself for 2 mL of blood meaning it is very time consuming for such a low throughput (especially when compared to the high throughput nature of inertial microfluidics). Once attached to the device surface cells are laser capture micro-dissected with 3 cells pooled for whole genome amplification followed by microarray analysis.[39]

While the majority of the research in the field of cell-based NIPT has been devoted to blood, trophoblastic cells can also be obtained from the endocervical canal. Although this route provides a likely lesser challenge than the isolation of fetal cell from blood considering the

larger number of trophoblastic cells present in the endocervical canal, high levels of enrichment is still required to allow for clinical translation. Samples from the endocervical canal have been recently used successfully to isolate[40] and perform NGS for genomic profiling from 5 weeks gestation.[41] Enrichment was performed using HLA-G MACS and testing was performed using whole genome/exome sequencing to identify chromosomal structural abnormalities and *de novo* mutations.[18] However, the patient acceptability of this approach is likely lower with comparison to peripheral blood sampling. Nevertheless, inertial microfluidics could be readily applied to enrich samples obtained from the endocervical canal although its performance in this setting remains to be determined.

Finally, an important consideration in the development of a circulating fetal enrichment technology is its compatibility with the downstream strategy used to test for the presence of genetic abnormalities. Technologies including RT-PCR, chromosomal FISH, whole genome sequencing and array comparative genomic hybridization (aCGH) have been used in the literature.[13, 17, 32, 42] Sequencing-based diagnostic testing is likely to be broadly clinically implemented in the near future and recent progress in the field allows for testing to be carried out from very small number of cells down to the single cell level. Indeed recent studies have demonstrated advanced genetic analysis for circulating fetal cells including aCGH and single cell NGS which have demontrated copy number differences for whole and sub-chromosmal abberations[14] and deep sequencing towards screening for monogenic disorders.[36]

To demonstrate the feasibility of inertial microfluidics with sequencing-based testing, we developed a methodology to pick-up single fetal trophoblastic cells. To this end, the enriched sample was stained for cytokeratin (KRT), CD45 and DAPI and automated microscopy was used to identify the trophoblastic cells. We have also shown that cells that have been processed with this method can be used to carry out genetic testing, and in this case we have used the

Veriseq protocol used for preimplantation genetic screening approach utilizing Illumina MiSeq which can determine chromosomal copy number. Although in this instance the NGS profile was noisy which may be influenced by stage of cell cycle or cell degradation it must be noted that sample aligned to the human genome with >350,000 reads and due to the fact that chromosome Y was detected indicates JEG3 cells were successfully isolated, amplified and sequenced from a female spiked blood sample.

3. Conclusion

The data presented in this study demonstrates that inertial microfluidics provides an efficient yet simple and gentle method to isolate rare fetal cells from maternal blood. Although this approach should be validated in a clinical study, inertial microfluidics possesses many advantages over standard enrichment approaches towards the isolation of circulating fetal cytotrophoblast and SNA as has been demonstrated in this feasibility study from maternal blood at 11 weeks gestation. Most notably, inertial microfluidic technology provides high throughput, rapid and gentle isolation that is not reliant on antibody expression. We have also shown that inertial microfluidics separation is amenable to downstream genetic analysis with a commercially available single cell preimplantation genetic screening approach.

The development and validation of a clinical technology for the efficient isolation of circulating fetal trophoblasts would foster the development of novel truly non-invasive prenatal test, with a strong potential to improve on the emerging gold standard method based on cffDNA including elimination of the maternal contribution which can influence current NIPT technology. Further development of this technology could not only eliminate the risks associated with current invasive procedures, but also provide reassurance to the large majority of low risk pregnancies.

4. Experimental Section

Characterization of Inertial Microfluidics

To optimize and enable accurate detection of the enrichment yields, MCF-7 breast cancer cell lines and JEG3 a Human choriocarcinoma cell line (ECACC) stained with calcein (Sigma Aldrich, St. Louis, MO., USA) were used to mimic large trophoblastic cells. The cancer cell lines was cultured in 25 or 75cm^2 tissue culture flasks (Sarstedt, Nümbrecht Germany) in DMEM or AMEM supplemented with fetal bovine serum and penicillin/streptomycin (Sigma Aldrich, St. Louis, MO., USA) at 37^{o}C, 5% CO_2 in a humidified environment. Cancer cells were detached from culture flasks with trypsin/EDTA (Sigma Aldrich, St. Louis, MO., USA) and spiked into healthy blood samples. Healthy peripheral blood was collected from volunteers (in compliance with the University of South Australia Human Research Ethics Committee) into an EDTA vacutainer, and used within 24 hours after collection. Cell preparation and isolation were performed as outlined in the following sections. Cell recovery was quantitated with imaging flow cytometry based on the recovery of calcein positive events (cancer cells) and calcein negative events (WBC).

Sample Collection and Ethical Considerations

Ethics approval for sample collection (maternal peripheral blood) was approved by the Women's and Children's Human Research Ethics committee (WCH Application No: WEC/13/WCHN 45). Written informed consent was obtained from the patient prior to sample collection. It is important to note patients were enrolled in this study before undergoing amniocentesis, and that these patients are at an increased risk of genetic abnormalities. Maternal peripheral blood (14 mL) sample was taken just before amniocentesis (at approximately 11 weeks' gestation), by a trained nurse and was placed into K3EDTA Vacutainers and kept on ice or at 4^{o}C until use. All samples were processed within 6 hours after collection. To eliminate epithelial contamination commonly seen in blood drawn by venipuncture, the first 2ml will be discarded. Healthy peripheral blood samples were collected

from healthy donors in compliance with the University of South Australia Human Research Ethics Committee.

Cell Preparation and Isolation

Chemical red blood cell lysis was performed at room temperature with a 1:10-1:25 blood to lysis solution (145mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA in MilliQ water; Sigma Aldrich) dilution. After 10 minutes of shaking, samples were centrifuged at 500g for 10 minutes, the cell pellet was then washed and resuspended in PBS (×2 dilution) and the cellular suspension was loaded in a syringe. From this point inertial microfluidics was used to further enrich the fetal cells obtained from the maternal sample. The suspension was then injected through tygon tubing with a syringe pump into the inertial microfluidic chips (1700 µL/min) using a standard syringe pump (KD Scientific Legato Syringe pump). Eluted suspension, enriched in trophoblastic cells, were then collected and processed for downstream analyses. The microfluidic chips was fabricated using micromilling at the SA and NSW nodes of the Australian National Fabrication Facility with each being subjected to a strict control quality protocol.

Fluorescence Staining and Imaging Flow Cytometry

For use with the imaging flow cytometer cells were stained with the nuclear stain DAPI (4',6-diamidino-2-phenylindole) and with fluorescently labelled fluorochromes Anti-Human CD45 PE-Cy5.5 or Alexa 488 and Anti-Pan-Cytokeratin (AE1/AE3) Alexa Fluor® 488 or 660 (eBioscience, San Diego, CA, USA) using a standard flow cytometry protocol previously described.[43] Imaging flow cytometry was performed using the ImageStream^x Mark II (AMNIS, Seattle, WA, USA) and analysis of the cellular population was performed with IDEAS software Version 6.1 (AMNIS, Seattle, WA, USA). Potential trophoblast cells were identified as DAPI positive, CD45 negative with cytokeratin staining. Events per sample were

acquired at 40× magnification. 3 healthy blood samples from non-pregnant women was also run to confirm there was no false positive KRT staining.

Fluorescence in situ Hybridization (FISH) and Laser Scanning Cytometry

A maternal blood sample from a pregnancy with Trisomy 21 was processed and the large cells were isolated with inertial microfluidics as described above (7 mL whole blood processed for FISH protocol). The trophoblast outlet sample was resuspended in hypotonic solution (0.05mol/L potassium chloride) and left at room temperature for 10 minutes. Carnoy's fixative (3:1 methanol/acetic acid) was then added to the sample and centrifuged at 1100 rpm for 5 minutes. Supernatant was removed and cells were resuspended completely in Carnoy's fixative. This was repeated twice more and the sample was then stored at 4^{o}C in Carnoy's fixative until further processing. After centrifugation again Carnoy's fixative was removed leaving behind approx. 100 µl total volume, cells were resuspended in this volume and then spread onto slides. Slides were left to air dry before probes (spectrum green, 21 spectrum orange, AneuVysion Multicolor DNA probe kit) were applied to the slide and a coverslip placed on top and sealed with rubber cement to avoid drying out. Samples were hybridized at 75^{o}C for 5 minutes, before being incubated at 30^{o}C overnight. Slides were washed with 0.4× Saline-sodium citrate buffer (SSC) with NP-40 (pH 7) at 75^{o}C for 2 minutes and then with 2xSSC with NP-40 (pH 7) for 1 minute. Slides were mounted with DAPI anti-fade (Vector Shield) and a cover slip added for imaging. Microscopy was carried out using the CompuCyte iCys™ Laser Scanning Cytometer using DAPI (blue), FITC (green) and Cy3/PE (red) channels for detection of nuclei, Chromosome 13 and Chromosome 21, respectively. Using the software and a compucolour6 filter DAPI positive events with three 21 signals were identified. Positive cells had three distinct and separate 21 chromosome signals inside the nucleus with a similar intensity. A sample from a male pregnancy and also a healthy sample from a non-pregnant

women were also run following the procedure as described above with 0 false positive events found.

Cell Retrieval and Genetic Analysis

As with characterization of inertial microfluidics JEG3 cell lines (XY-male) have been spiked into a healthy blood sample (XX-female). Sample is then enriched with inertial microfluidics, fixed with formalin, stained for KRT, CD45 and DAPI as per fluorescence staining method. Sample is then injected into a well with a thin PBS layer and oil on top as previously described.[44] KRT positive and CD45 negative cell well is then scanned with a Nikon TiE fluorescence microscope and KRT positive, CD45 negative events were identified and retrieved manually with a Cellsorter (Cellsorter Company for Innovations, Budapest, Hungary). Individual cells are placed into a 2µl of PBS (Cell Signaling Technology, Massachusetts, USA) and stored at -80°C until sequencing was undertaken. Briefly the cell sample was amplified using the Sureplex DNA Amplification kit (Illumina) which utilizes linker-adaptor PCR technology. Cells were lysed and extracted using lysis buffer and extraction buffer/enzyme. This was followed by pre-amplification and amplification as per manufacturer instructions. All temperature incubation steps were performed on a thermo-cycler (Simpliamp, Life Technologies, Thermofisher). For each round of amplification a positive female DNA control (15 pg genomic DNA in 2.5 µl 1x PBS) was used and the negative control was equal to 2.5 µl of PBS. Conformation of amplification was determined on agarose gel using the E-Gel®iBase™system (Invitrogen, Thermofisher). Following this chromosome copy number was determined using sequencing by synthesis technology (Veriseq, Illumina) which uses paired-end dual index sequencing on a Miseq (Illumina). Reads were aligned to the human genome and chromosome copy number status was determined using Bluefuse Multi software (Ilumina).[45]

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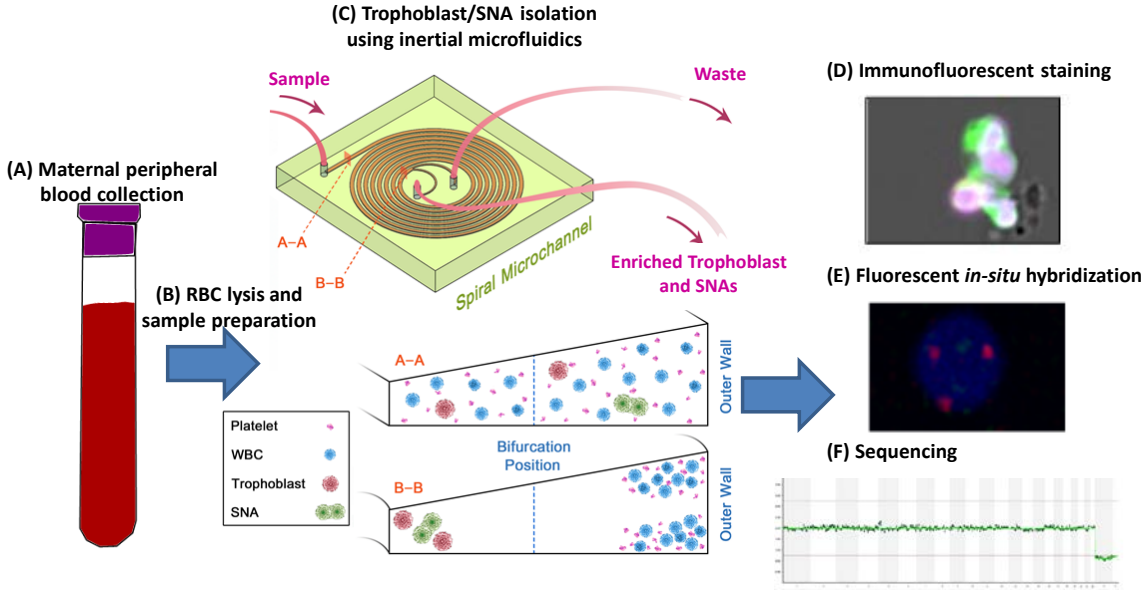

Figure 1 Schematic diagram of the experimental protocol followed when analyzing maternal blood for isolation and staining of fetal cells A) Maternal blood was collected and processed within 6 hours. B) Red blood cells were lysed and C) the nucleated cell fraction (nucleated fetal cells; white blood cell, WBC) was collected and resuspended in phosphate buffered saline (PBS). Samples were run through the inertial microfluidic device using a syringe pump at 1700µl/min. D) Trophoblast (inner) outlet was plated and stained with CD45, KRT (cytokeratin) and DAPI and imaged with imaging flow cytometry. E) Fluorescence *in situ* hybridization (FISH) was performed on the trophoblast outlet and imaged with a standard fluorescence microscope or Laser Scanning Cytometry. F) Example of a normal male cell isolated with this method and sequenced as a model of a trophoblastic cell.

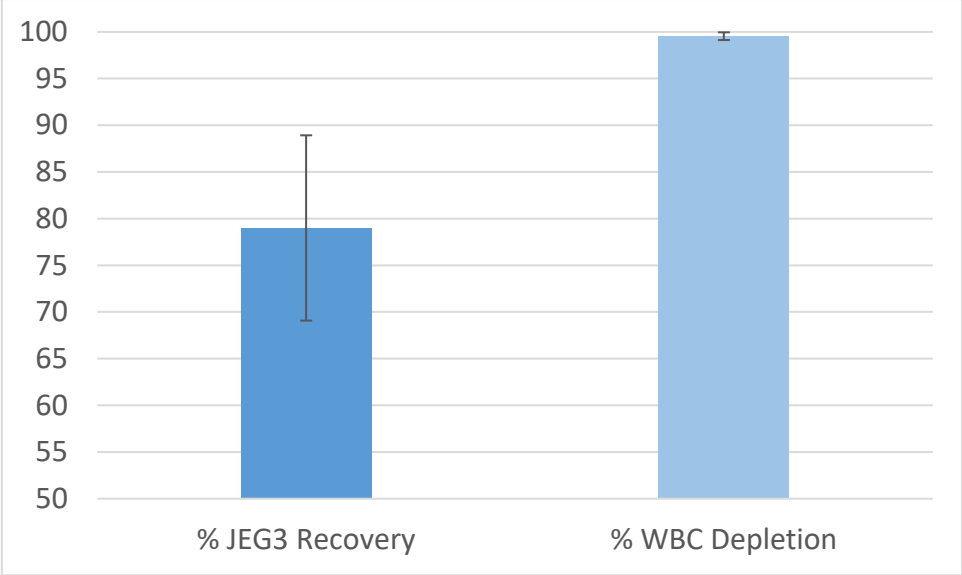

Figure 2 Bar graph depicting the recovery percent of JEG3 cells after inertial microfluidic enrichment and percent white blood cell (WBC) depletion of the enriched outlet after passing through the inertial microfluidics.

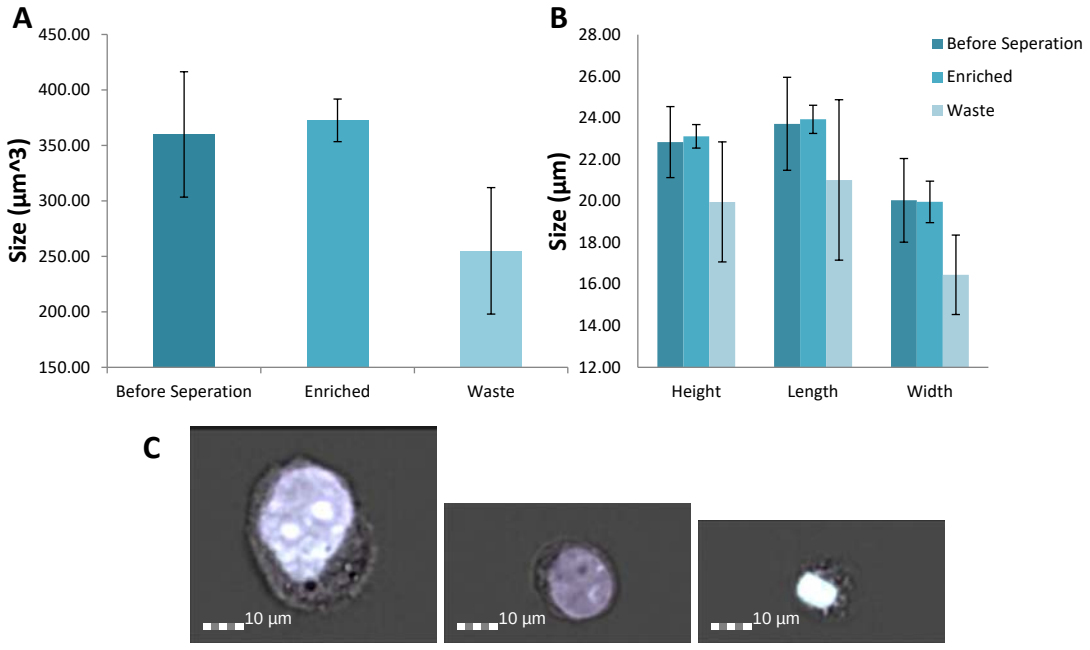

Figure 3 JEG3 size characteristics based on brightfield from the imaging flow cytometer with cells from before separation with inertial microfluidics or recovered after separation from the big cell or white blood cell (WBC) outlets. A) Graph of single cell area, B) Graph of single cell height, length and width. C) Imaging flow cytometry micrographs (composite image with brightfield and nuclei stained with Hoechst) of JEG3 cells demonstrating their large size variation.

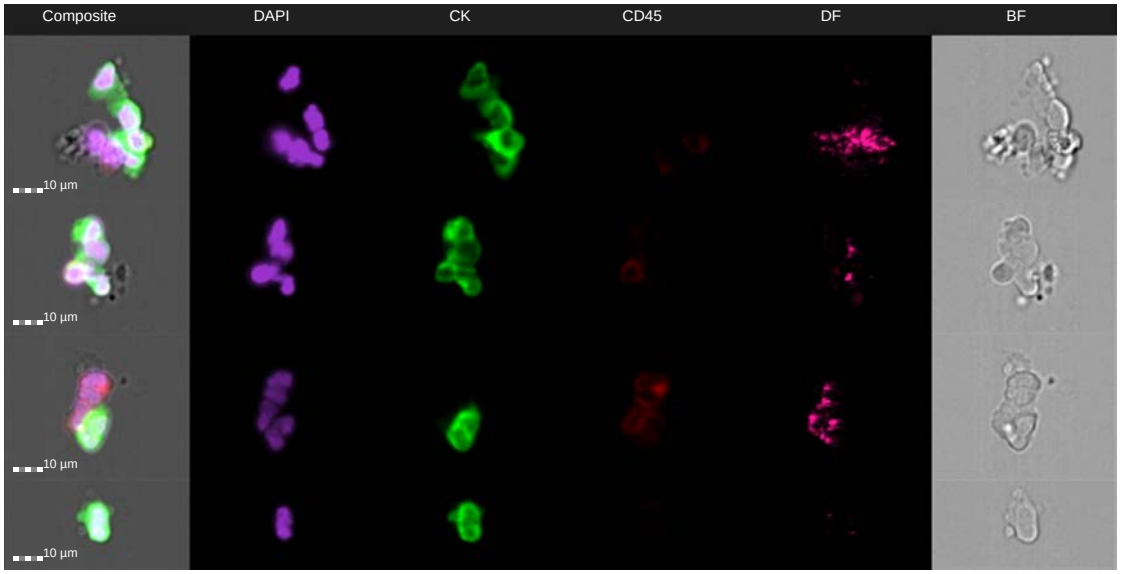

Figure 4 Trophoblastic images of cytokeratin positive events from the imaging flow cytometer from a maternal blood sample (Trisomy 21) that has undergone inertial microfluidic enrichment. Images have been adjusted to enhance visual appearance and were all taken at 40×magnification.

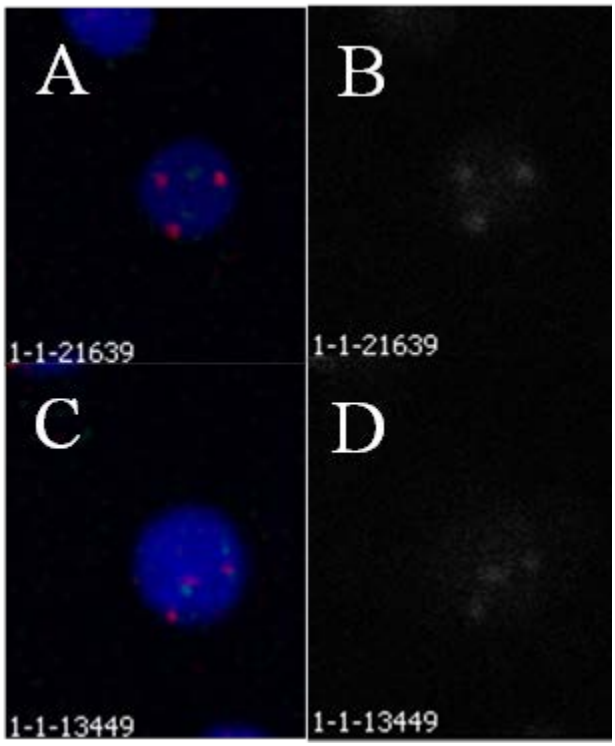

Figure 5 Fetal cells positive for Trisomy 21 (chromosome 21; red signal, and chromosome 13; green signal) identified from a maternal blood sample carrying a male fetus with diagnosed Trisomy 21 abnormality. A, C) Processed computed color image of fetal cells and B, D) raw fluorescent red signal.

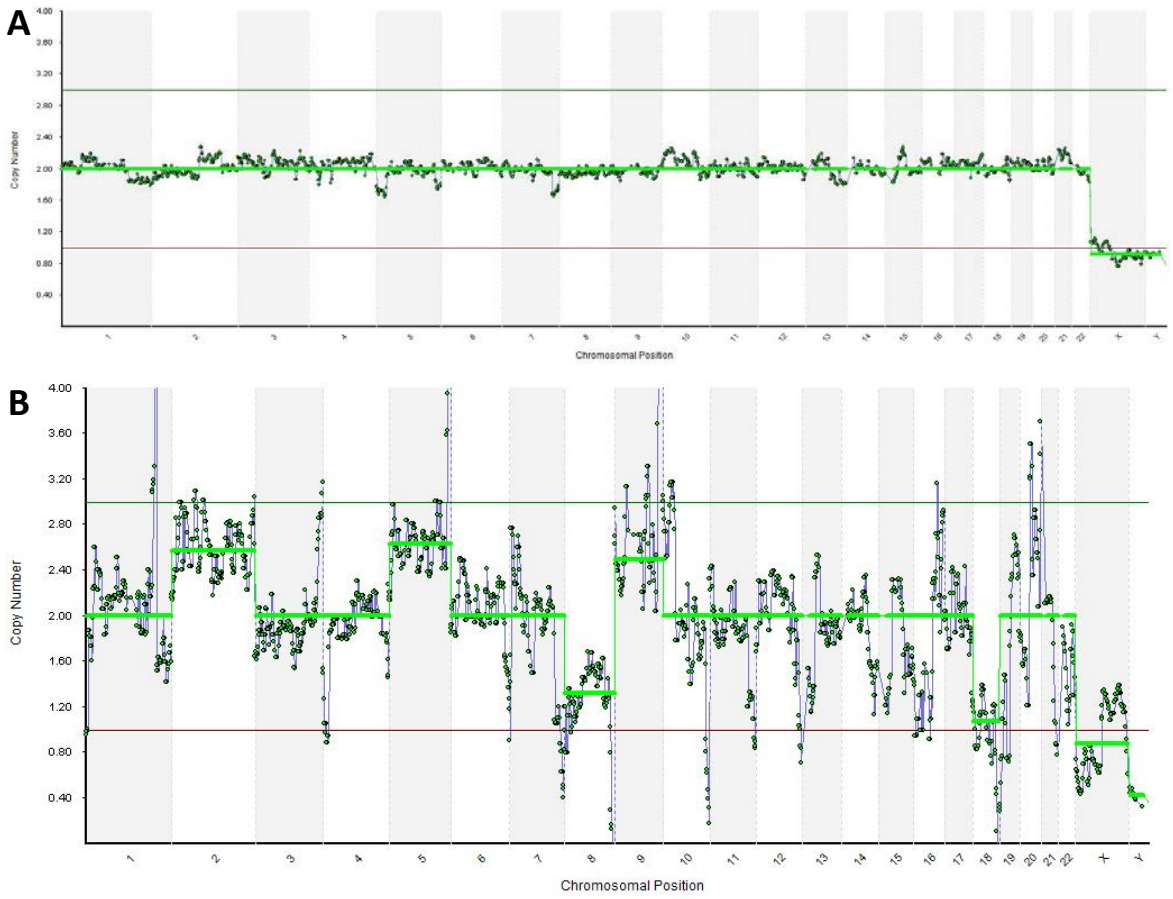

Figure 6 Individual cells are placed in a PCR tube and undergo sequencing with Illumina MiSeq. Profile from individual normal male cell (A) and an abnormal JEG3 cell hypertriploid profile (B).

Table 1. Cytokeratin Positive Trophoblast Characteristics from Trisomy 21 affected pregnancy

	Average	St Dev	Min	Max
Area (μm^2)	321	150	168	468
Length (μm)	27.2	10.5	16.5	37.5
Width (μm)	18.7	6.43	14	26