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(54) Title: PRE-NATAL CELL ISOLATION

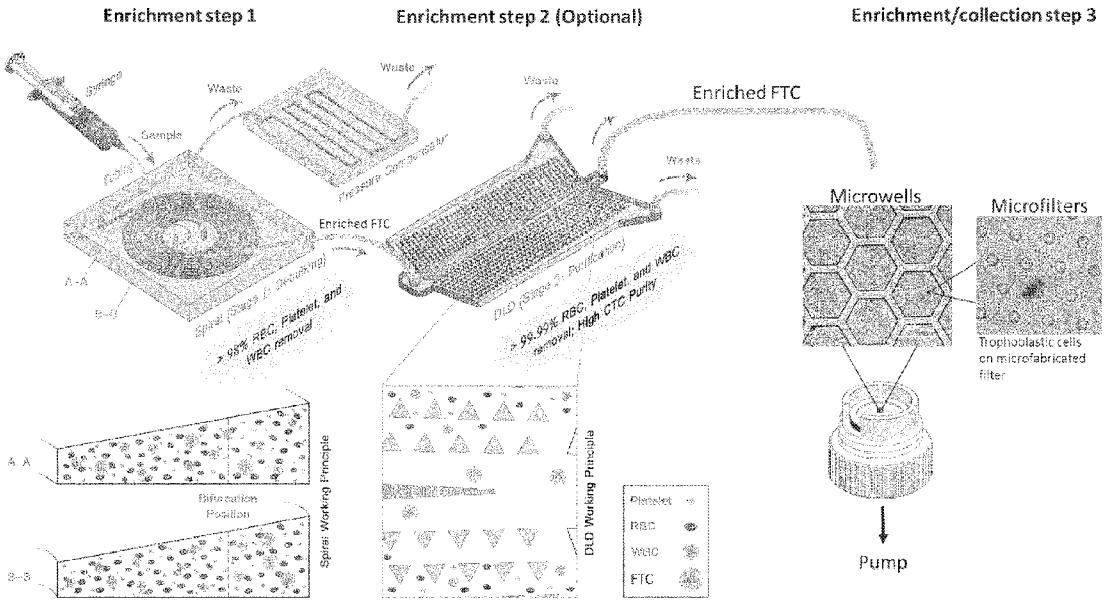

Figure 1

(57) Abstract: A method of isolating fetal trophoblastic cells from a suitable biological sample is disclosed. The method comprises: providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types; enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population; and collecting isolated fetal trophoblast cells by applying the enriched fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

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PRE-NATAL CELL ISOLATION

PRIORITY DOCUMENT

[0001] The present application claims priority from Australian Provisional Patent Application No. 2018900373 titled "PRE-NATAL CELL ISOLATION" and filed on 7 February 2018, the content of which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] The present disclosure relates to methods of isolating cells. In a particular form, the present disclosure relates to methods of isolating fetal cells.

BACKGROUND

[0003] Prenatal diagnosis of chromosomal abnormalities is traditionally performed using invasive procedures, including chorionic villus sampling (CVS) and amniocentesis. These invasive procedures cannot be carried out early in pregnancy (eg before approximately 10 weeks) and are associated with a significant risk of miscarriage. These limitations have driven the development of non-invasive prenatal testing (NIPT) assays. NIPT based on circulating cell-free fetal DNA (cffDNA) found in maternal blood has gained broad clinical acceptance for the detection of common aneuploidies. cffDNA originates from apoptotic cells in the placenta and is found at various levels in maternal blood. The current false-positive rate in NIPT for common aneuploidies is approximately 0.1%–0.2%, even for low risk pregnancies. The American College of Obstetrics and Gynaecology and the International Society of Prenatal Diagnosis advocate the implementation of cffDNA screening in high-risk pregnancies.

[0004] However, cffDNA-based NIPT remains mainly a screening technology and invasive testing is still required for a conclusive diagnosis to guide clinical decision-making as current NIPT technologies do not have the capability to provide information about the full range of chromosomal abnormalities and single gene disorders. In addition, to date, cffDNA NIPT detects only a small subset of the chromosomal abnormalities that can be diagnosed using standard invasive procedures. Further, the potential for whole genome fetal sequencing using cell-free fetal DNA is limited due to the high proportion of maternal cell-free DNA in maternal plasma (>90%) and the inability of current technologies to determine the origin of cell free DNA fragments using bioinformatic techniques. In addition, cffDNA mainly consists of fragmented short DNA which limits the depth of interrogation of the genome. Although certain modes of inheritance (paternally-derived autosomal dominant disorders and *de novo* fetal mutations) could theoretically be detected using cell free DNA analysis, there is no clinically viable, universal protocol for fetal genomic profiling.

[0005] By contrast, isolated fetal cells have the potential for whole genome fetal sequencing using protocols initially developed for preimplantation genetic diagnosis. The possibility of isolating intact fetal cells from maternal blood as a source of fetal genomic DNA for prenatal diagnosis has been tantalizing since the implementation of routine prenatal diagnostic testing, as such cells could potentially provide advantageous means for performing NIPT assays compared to cffDNA based NIPT, and, ultimately to conventional cytogenetic methods. However, fetal cells in maternal circulation are exceedingly rare, making the isolation and/or genetic analysis of these cells a tremendous challenge. Attempts have been made to isolate fetal cells from maternal circulation, including density gradient centrifugation, fluorescence activated cell sorting, magnetic activated cell sorting, magnetophoresis and dielectrophoresis. However, these techniques all suffer from significant drawbacks including low efficiencies and low specificities, and have failed to provide a valid alternative to invasive sampling or cffDNA approaches.

[0006] It is desirable to provide a non-invasive method able to isolate fetal cells from suitable biological specimens that is potentially compatible with downstream cytogenetic, genomic, proteomic or transcriptomic assays.

SUMMARY

[0007] According to an aspect of the present disclosure, there is provided a method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b.i) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population;

(b.ii) optionally purifying the enriched fetal trophoblast cell population to a deterministic lateral displacement (DLD) device to obtain a purified fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying a cell population selected from the enriched fetal trophoblast cell population or the purified fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

[0008] According to an aspect of the present disclosure, there is provided a method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b.i) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device optimised to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population;

(b.ii) purifying the enriched fetal trophoblast cell population by applying the enriched fetal trophoblast cell population to a deterministic lateral displacement (DLD) device to obtain a purified fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying the purified fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

[0009] According to an aspect of the present disclosure, there is provided a method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying the enriched fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

[0010] In certain embodiments, the step of enriching the fetal trophoblastic cell population is performed two, three or more times to further enrich the fetal trophoblast cell population.

[0011] In certain embodiments, the suitable biological sample is a maternal biological fluid sample. In certain embodiments, the suitable biological sample is selected from maternal blood, maternal cervical mucus, maternal endocervical canal, maternal endometrial cavity, from lavage of the maternal endocervical canal or the uterine cavity, and endometrial biopsy.

[0012] In certain embodiments, following step (c), each microwell within the array of microwells collects an average of one or fewer cells. In another embodiment, following step (c), each microwell within the array of microwells collects an average of one or more cells. Where one or more cells are collected in a microwell, serial dilution may be performed.

[0013] In certain embodiments, the array of microwells further comprises a microfilter having a porosity that retains fetal trophoblast cells and allows other cell types to pass through.

[0014] In certain embodiments, the array of microwells further comprises a microfilter having a porosity that retains fetal trophoblast cells and other cell types to allow removal of excess liquid to concentrate the fetal trophoblast cells and other cell types.

[0015] In certain embodiments, the microfilter is coated with an agent to reduce background autofluorescence and improve fluorescent imaging of fetal trophoblast cells.

[0016] In certain embodiments, the microfilter is functionalised with a binding partner that specifically binds to cellular receptors of trophoblastic cells.

[0017] In certain embodiments, the method further comprises the following step:

(d) staining the collected cells to identify isolated fetal trophoblastic cells.

[0018] In certain embodiments, the isolated fetal trophoblastic cells are used for cytogenetic, genomic, proteomic, transcriptomic and/or metabolomic assays. For example, the isolated fetal trophoblastic cells may be assessed for genetic or chromosomal abnormalities and/or the isolated fetal trophoblastic cells may be used for proteomic, transcriptomic or metabolomic analysis for the detection of pregnancy complications, such as placental dysfunctions.

[0019] According to an aspect of the present disclosure, there is provided a method of prenatally diagnosing a genetic or chromosomal abnormality or a placental dysfunction in a fetus comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells.

[0020] According to an aspect of the present disclosure, there is provided a method for cytogenic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing genetic material from the isolated fetal trophoblast cells.

[0021] According to an aspect of the present disclosure, there is provided a method for genomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing genetic material from the isolated fetal trophoblast cells.

[0022] According to an aspect of the present disclosure, there is provided a method for proteomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells.

[0023] According to an aspect of the present disclosure, there is provided a method for transcriptomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells

from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing genetic material from the isolated fetal trophoblast cells.

[0024] According to an aspect of the present disclosure, there is provided a method for metabolomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells.

BRIEF DESCRIPTION OF DRAWINGS

[0025] Embodiments of the present disclosure will be discussed with reference to the accompanying drawings wherein:

[0026] Figure 1 is a schematic diagram showing an embodiment of the method of the present disclosure;

[0027] Figure 2 is an example of a typical scatter plot of calcien intensity against bright field (BF) cellular area from (A) a pre-sample and (B) a sample obtained from the trophoblast outlet of an inertial microfluidic device. (C) Image of JEG3 stained cells, and (D) unstained white blood cells. Images taken at 400× magnification and have been adjusted to enhance visual appearance;

[0028] Figure 3 shows micrographs of HLA-G and Hoechst positive, CD45 negative fetal trophoblastic cell (green box) from a pregnant maternal blood sample after separation with an inertial microfluidic device. (A) Hoechst, (B) HLA-G, (C) CD45 (100x magnification), and (D) brightfield (400x magnification);

[0029] Figure 4 shows photos of gold coated microwell microfilters with (A) 150µm wells and (B) 500µm wells taken before functionalization with HLA-G antibody to specifically bind trophoblastic cells. SU8 filters were coated with a 25-100nm gold layer then functionalised with 11-Mercaptoundecanoic acid (MUA) or polyethylene glycol (PEG) before attaching the HLA-G antibody;

[0030] Figure 5 shows micrographs of single stained JEG-3 (primary HLA-G antibody with secondary Anti-mouse IgG FITC antibody and Hoechst) in 500 µm well (A) and stained JEG-3 (Cytokeratin FITC and DAPI) in 220 µm honeycomb well (B). Detection of HLA-G or Cytokeratin and a nuclear signal indicates the presence of a fetal trophoblastic cell, the cell can then be picked up or lysed with a manual pipette or single cell manipulator system;

[0031] Figure 6 shows micrographs of JEG3 cells within a microwell (in a thin layer of media with mineral oil on top to facilitate for pick up) before (A) and after pick up (B) with a 30µm micropipette; (C) shows that 1 cell was picked up and this was deposited into a PCR tube for downstream analysis (100×magnification); (D) cells deposited onto a glass slide (400× magnification). The positive deposition of a single cell visualised with fluorescence microscopy;

[0032] Figure 7 shows a cytokeratin positive, DAPI positive cytotrophoblast cell isolated from a pregnant woman's blood sample before (A) and after pick up (B). The sample was stained for Cytokeratin, DAPI and CD45 (APC). Image taken at 400× magnification.

[0033] Figure 8 is micrograph of a microfilter with 500µm wells (brightfield) and an 80µm single cell manipulation micropipette (red circle) able to pick up cells within the well, and dispense/remove lysis buffer/lysed solution respectively;

[0034] Figure 9 shows micrographs of a single JEG3 cell (red circle) lysis within the microwell, before (A) and after lysis (B) within a 150 µm microwell; (C) Visual representation demonstrating single well buffer manipulation (in this case red dye), in order to specifically target and lyse 1 microwell;

[0035] Figure 10 shows a single stained cell (Hoechst nuclear stain and calcein) before (A) and after (B) lysis in a 500µm honeycomb microwell. An example of a droplet digital PCR profile for KRT19 (Keratin 19), in this instance multiple cells were lysed on filter, the mRNA was recovered, converted to cDNA before undergoing PCR (QX200™ droplet digital PCR system BioRad);

[0036] Figure 11 is a micrograph of multinucleated syncytial nuclear aggregate (SNA) stained with May-Grunwald Giemsa (400x magnification) (A), and SNAs identified as being keratin positive (pan-KRT) and CD45 negative by imaging flow cytometry (400x magnification) (B);

[0037] Figure 12 shows micrographs of a CD45 negative, Hoechst Positive syncytial nuclear aggregate from a pregnant maternal blood sample (where the pregnancy was complicated with pre-eclampsia) after separation with inertial microfluidic device. (A) Brightfield before picking, (B) Hoechst before picking, (C) CD45 FITC before picking (400× magnification), and (D) Hoechst after picking demonstrating efficient pick up of the designated cell (100× magnification);

[0038] Figure 13 (A, C) shows an example of a computed colour image of a cytotrophoblast with fluorescence *in situ* hybridisation probing for chromosomes 13 (green) and 21 (red); and (B, D) Raw red imaging of chromosome 21 signals in the same cell as A and C, respectively;

[0039] Figure 14 shows examples of fluorescence *in situ* hybridisation of circulating fetal cells from 3 pregnancies with male fetuses enriched with inertial microfluidics (DAPI nuclear stain , Y chromosome: + (DYZ3), X chromosome: * (DXZ1)). Images taken at 400× magnification;

[0040] Figure 15 shows an example of an isolated circulating fetal cell which after enrichment was stained with specific markers and DAPI nuclear staining (A) and placed in a microwell. This cell was then recovered and placed onto a slide for fluorescence in situ hybridisation for X (*) and Y (+) chromosomes confirming that this fetal cell was from a male fetus (B); and

[0041] Figure 16 shows an example of a water (represented by #) in oil (represented by *) droplet containing a single cell (circle), Brightfield image (A) and nuclear DAPI image (B) of the same droplet attached to a glass surface. An image of a microneedle (arrow) depositing a water droplet (C) and of a water droplet containing multiple cells which was free floating in the oil droplet (D).

DESCRIPTION OF EMBODIMENTS

[0042] The present disclosure relates to a method for the isolation of fetal trophoblastic cells from biological samples such as fluids, including maternal blood and cervical mucus, using a combination of microfluidic technologies. The method provides isolated fetal cells that can potentially be used for non-invasive prenatal diagnosis using cytogenetic, genomic, proteomic or transcriptomic methods.

[0043] Fetal cells including lymphoid and erythroid cells, myeloid precursors, and trophoblastic (epithelial) cells are found in maternal circulation in very low numbers (estimated to be ~1-2 trophoblasts per mL of blood; Mouawia *et al.*, 2012). Extravillous trophoblast cells isolated using ISET ("Isolation by Size of Epithelial Tumor Cells" described in Patent US7651838) were consistently larger than 15 μm. Trophoblasts could be detected as early as 5 weeks gestation and notably were also found not to persist post-termination/birth. Syncytial nuclear aggregates (SNAs) 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. Elevated numbers of placental extravillous trophoblastic cells and SNAs are observed in pregnancy complications including preeclampsia, reduced fetal movements, intra-uterine growth restriction and stillbirth (Correa *et al.*, 2008). Trophoblastic cells can also be found in the uterine cavity and a recent study demonstrated the feasibility of using syncytiotrophoblasts isolated from the cervical mucus for prenatal diagnostic (Mantzaris and Cram, 2015). Accordingly, isolation of fetal trophoblasts or SNAs would be useful in non-invasive diagnostic techniques.

[0044] The present disclosure relates to an isolation method for fetal trophoblasts from suitable biological samples using a series of efficient separation techniques. In certain embodiments, fetal

trophoblasts are larger than many other cell types, including white blood cells. However, an extraordinarily efficient isolation procedure is required to isolate fetal trophoblast cells from suitable biological samples containing these cells with the level of purity required for most diagnostic assays. In certain embodiments, the present disclosure relates to sequential application of microfluidic technologies aimed at isolating fetal trophoblastic cells. The identity of these cells can then optionally be confirmed (eg using trophoblast cell markers), and potentially assessed for diagnostic purposes. In certain embodiments, SNAs may alternatively or additionally be used in the method disclosed herein in place of fetal trophoblastic cells.

[0045] Accordingly, in an aspect, the present disclosure provides a method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising a fetal trophoblastic cell population within a population of other cell types;

(b.i) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device optimised to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population;

(b.ii) optionally purifying the enriched fetal trophoblast cell population by applying the enriched fetal trophoblast cell population to a deterministic lateral displacement (DLD) device to obtain a purified fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying a cell population selected from the enriched fetal trophoblast cell population or the purified fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

[0046] In an aspect, the present disclosure provides a method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b.i) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device optimised to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population;

(b.ii) purifying the enriched fetal trophoblast cell population by applying the enriched fetal trophoblast cell population to a deterministic lateral displacement (DLD) device to obtain a purified fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying the purified fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

[0047] In an aspect, the present disclosure provides a method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying the enriched fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

[0048] In certain embodiments, the suitable biological sample comprises a fetal trophoblastic cell population within a population of other cell types. In certain embodiments, the suitable biological sample is a maternal biological sample, that is, the sample is derived from a pregnant female, in order to isolate fetal trophoblasts derived from the fetus of the current pregnancy. In certain embodiments, the suitable biological sample is a maternal biological fluid. In certain embodiments, the suitable biological sample is maternal blood, cervical mucus or lavage fluid of the endometrial cavity obtained during the pregnancy; however, it is envisioned that other types of biological sample may be suitable for use in the method of the present disclosure. In certain embodiments, the biological sample is derived from cotton swabs or a cytobrush of the endocervical canal and the endometrial cavity, from lavage of the endocervical canal or the uterine cavity, from aspiration of cervical mucus, or from endometrial biopsy using, for example, a Pipelle. In certain embodiments, the cells derived from the sample may be resuspended in a suitable fluid such as sterile buffers or media well known to those skilled in the art. In certain embodiments, the biological sample is maternal blood. In certain embodiments, the biological sample is derived from a human female; however it is anticipated that the method of the present disclosure could easily be adapted for use in pregnant non-human animals of interest or commercial value.

[0049] In certain embodiments, the sampling is performed in the first trimester of the pregnancy. However, it is envisioned that the sampling could alternatively occur during the second or third trimester of the pregnancy. In certain embodiments, the sampling occurs before the 16^{th} week of the pregnancy. In certain embodiments, the sampling occurs before the 14^{th} week of the pregnancy. In certain embodiments, the sampling occurs before the 12^{th} week of the pregnancy. In certain embodiments, the sampling occurs before the 10^{th} week of the pregnancy. In certain embodiments, the sampling occurs before the 8th week of the pregnancy. In certain embodiments, the sampling occurs before the 6^{th} week of the pregnancy. The week of the pregnancy can be determined using conventional techniques which are well known in obstetrics.

[0050] In certain embodiments, the biological sample is treated to fix the cells in the sample prior to application of the sample to the inertial microfluidic device. As would be well understood by the person skilled in the art, fixation of cells in the biological sample can be carried out using any of the reagents and methods known for that purpose in the art, such as by treatment with a suitable fixative. By

way of example, suitable chemical fixatives can be selected from one or more of the group consisting of formaldehyde (eg. formalin), glutaraldehyde, methanol and ethanol.

[0051] In certain embodiments, the method comprises an initial sample processing step. In certain embodiments, the initial sample processing step (eg an enrichment step) enriches the population of fetal trophoblastic cells by eliminating other cells including red blood cells, white blood cells, tissue cells and/or cellular debris. In certain embodiments, the enrichment is through density gradient methods. In certain embodiments, the enrichment step is a density gradient method that uses dextran using a method described by Lin *et al* (2017), the disclosure of which is hereby incorporated in its entirety. In certain embodiments, the initial processing step isolates the nucleated fraction of the sample using standard red blood cell lysis methods.

[0052] In certain embodiments, the inertial microfluidic device is a spiral device. A spiral device mediates cell separation based on size and deformability of the cells, relative to other cells and components in the sample, typically with larger cells along the inner wall and the smaller cells away from the inner wall. Suitable inertial microfluidic devices include Clearbridge BioMedics CTChip®FR (Clearbridge BioMedics Pte Ltd, Singapore), and devices described in PCT/US2011/027276 (WO 2011/109762), PCT/SG2013/000412 (WO 2014/046621) or PCT/US2013/024403 (WO2013116696), the disclosures of which are hereby incorporated in their entirety. In another embodiment, the inertial microfluidic device is a vortex device. Suitable vortex devices include devices described in US9133499 B2, the disclosure of which is hereby incorporated in their entirety. However, it is anticipated that other devices may also be suitable for use in the method of the present disclosure to enrich the population of fetal trophoblastic cells.

[0053] In certain embodiments, the inertial microfluidic device is modified to optimise separation of fetal trophoblastic cells from other cell types in the biological sample. Such modifications may include optimising the pressure within the inertial microfluidic device (eg using a pressure compensating device), altering the length of the channels used in the device, altering the shape and/or dimensions of the channels within the device, altering the geometry of cross section (eg trapezoidal), altering the outlet position (which alters resistance), altering the shape of the device, altering the angle of the channels (eg the curvature of the spiral), altering the viscosity of the medium, altering the length and height of tubing (eg relative to a syringe pump) etc, as would be well understood by a person skilled in the art.

[0054] In certain embodiments, the biological sample is run through the inertial microfluidic device at a rate of at least 100 microlitres per minute (μL/min), for example, at a rate between approximately 100 μL/min and 5000 μL/min, or 1000 μL/min and 3000 μL/min, such as 1300 μL/min, 1400 μL/min, 1500 μL/min, 1600 μL/min, 1700 μL/min, 1800 μL/min, 1900 μL/min, 2000 μL/min, 2100 μL/min, 2200 μL/min, 2300 μL/min, 2400 μL/min or 2500 μL/min. In certain embodiments, the inertial microfluidic

device comprises 1, 2, 3 or more inlets. In certain embodiments, the inertial microfluidic device comprises 1, 2, 3 or more outlets. In certain embodiments, the channel cross section has a width of between 50 μm and 1000 μm, for example, between 300 μm and 800 μm, such as between 400 μm and 700 μm or 500 μm and 600 μm. In certain embodiments, the channel cross section has a height of between 20 μm and 500 μm, for example, between 30 μm and 300 μm, such as between 50 μm and 200 μm or 80 μm and 160 μm. The channel may have any suitable cross section, for example, circular, elliptical, rectangular, equilateral triangular, non-equilateral triangular, irregular quadrilateral, trapezoidal or teardrop with vertex cut off. In certain embodiments where the geometry of the channel cross section allows (eg irregular quadrilateral, trapezoidal, teardrop with vertex cut off etc), the channel may have different inner and outer heights which may increase the efficiency of cell migration and focusing. An inner height to outer height ratio may be between about 19:20 to about 1:4, for example, between about 10:11 to about 1:3 or between about 9:12 to about 1:2. In certain embodiments, an inner height may be, for example, between 70 μm and 90 μm and an outer height may be between 120 μm and 140 μm. In certain embodiments, a path length of the inertial microfluidic device is between 50 mm and 200 mm, for example, between 60 mm and 180 mm, such as between 70 mm and 150 mm or 80 mm and 120 mm. As would be appreciated by the person skilled in the art, the channel dimensions may be selected such that only the larger cells undergo inertial focusing, while migration of the smaller blood cells is solely affected by the Dean drag. In certain embodiments, the inertial microfluidic device comprises a spiral of between 5 and 12 loops, for example, between 6 and 10 loops, such as between 7 and 9 loops. As would be appreciated by the person skilled in the art, altering the radius of the inertial microfluidic device may provide for more efficient cell migration and focusing. In certain embodiments, a radius of the spiral increases. For example, the radius may increase by any amount between about 1mm to about 100 mm, for example, from about 2 mm to about 60 mm, or from about 3 mm to about 50 mm, or from about 4 mm to about 40 mm, such as about 5 mm to about 30 mm or about 8 mm to about 24 mm. In alternative embodiment, a radius of the spiral decreases. For example, the radius may decrease by any of the above described amounts by which the radius increases.

[0055] Methods for fabricating the inertial microfluidic device are known in the art. For example, the device can be fabricated using standard photolithographic and etching procedures including soft lithography techniques (e.g. see Shi J., et al., Applied Physics Letters 91, 153114 (2007); Chen Q., et al., Journal of Microelectromechanical Systems, 16, 1 193 (2007); or Duffy et al., Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane), Anal. Chem., 70 (23), 4974-4984 (1998)), such as near-field phase shift lithography, microtransfer molding, solvent-assisted microcontact molding, microcontact printing, and other lithographic microfabrication techniques employed in the semiconductor industry. Direct machining or forming techniques may also be used as suited to the particular chip. Such techniques may include hot embossing, cold stamping, injection moulding, direct mechanical milling or micromilling, laser etching, chemical etching, reactive ion etching, physical and chemical vapour

deposition, and plasma sputtering. The particular methods used will depend on the function of the particular microfluidic network, the materials used as well as ease and economy of production. Suitable materials may include, but are not limited to, polydimethylsiloxane (PDMS), thiol-ene polymers, UV-curable optical adhesives such as NOA81 or UV-curable epoxy resin based photoresists such as SU-8.

[0056] In a particular embodiment, the inertial microfluidic device comprises 2 inlets and 2 outlets having a channel that has a width of about 500 μm and a height of about 160 μm with a total path length of about 100 mm, and where the device is fabricated with photolithography and comprises of PDMS bonded to a glass slide. In another particular embodiment, the inertial microfluidic device comprises an 8-loop spiral microchannel with one inlet and two outlets with the radius increasing from about 8 mm to about 24 mm, where the width of the channel cross-section is about 600 μm, and the inner/outer heights are about 80 μm and about 130 μm, respectively, for the trapezoid cross-section, and where the device is fabricated through micromilling and soft lithography and comprises PDMS bonded to PDMS.

[0057] In certain embodiments, the step of enriching the population of fetal trophoblastic cells (enrichment step) utilises an inertial microfluidic device that debulks the biological sample. In certain embodiments, the enriching step removes > 80% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 85% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 90% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 95% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 96% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 97% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 98% of the other cell types from the biological sample. In certain embodiments, the enriching step removes > 99% of the other cell types from the biological sample. In certain embodiments, the other cell types removed from the biological sample include cells selected from red blood cells, platelets, white blood cells, other maternal cells, cellular debris etc. In certain embodiments, the enriching step is performed two, three or more times to further enrich the fetal trophoblast cell population.

[0058] In certain embodiments, the inertial microfluidic device is coupled with a pressure compensator device attuned to the fluidics required to bring the enriched fetal trophoblastic cell sample to the following device used in the method, that is, either the DLD device in embodiments where a secondary (purifying) step is performed, or to the microwell array device, as described herein. Examples of the pressure compensator device would be known to those skilled in the art.

[0059] In certain embodiments, the enriched fetal trophoblast cell population obtained from inertial microfluidic device is treated to fix the cells in the sample. As would be well understood by the person skilled in the art, fixation of cells in the biological sample can be carried out using any of the reagents and

methods known for that purpose in the art, such as by treatment with a suitable fixative. By way of example, suitable chemical fixatives can be selected from one or more of the group consisting of formaldehyde (eg. formalin), glutaraldehyde, methanol and ethanol.

[0060] In certain embodiments, the method of the present disclosure optionally comprises a second step that is a purification step. Such a purification step may be mediated by a deterministic lateral displacement (DLD) device; although it is anticipated that other devices may suitably purify the fetal trophoblastic cells. In certain embodiments, examples of suitable deterministic lateral displacement (DLD) device may be selected or adapted from those described in Huang *et al.*, 2004; Davis *et al.*, 2006; PCT/US2010/032150 (WO2010124155); the disclosures of which are hereby incorporated in their entirety.

[0061] In certain embodiments, following the purifying step, more than 90% of the other cell types are removed from the biological sample. In certain embodiments, following the purifying step, more than 95% of the other cell types are removed from the biological sample. In certain embodiments, following the purifying step, more than 98% of the other cell types are removed from the biological sample. In certain embodiments, following the purifying step, more than 99% of the other cell types are removed from the biological sample. In certain embodiments, following the purifying step, more than 99.9% of the other cell types are removed from the biological sample. In certain embodiments, following the purifying step, more than 99.99% of the other cell types are removed from the biological sample.

[0062] In certain embodiments, isolated fetal trophoblastic cells are collected following the enriching step and optionally following the secondary purifying step by applying a cell population selected from the enriched fetal trophoblast cell population or the purified fetal trophoblast cell population to a microwell array to collect isolated fetal trophoblast cells. In certain embodiments, suitable microwell arrays have microwells with a diameter range between about 10 to 1000 μm or about 50 to 1000 μm, although other diameters may be suitable, eg in the range of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1200 1500, 2000 or 5000 μm. The microwell arrays may have any suitable well design, for example, square or honeycomb. Suitable microarrays would be known to persons skilled in the art.

[0063] In certain embodiments, the microwell array may be in the form of water droplets in oil wherein cells are deposited into a water droplet within the oil. Water droplets in oil can be formed using a micromanipulator or by manual pipetting. In these embodiments, the water droplets behave in the same way as microwells allowing containment of the cell or lysis/amplification within the droplet. As such, use of the term "microwell array" or similar terms herein is intended to include classical microwell arrays and functional equivalents of them, such as water droplets in oil. Also, the above approach using droplets can be used on the filter. Accordingly, amplification of DNA or RNA may occur on the filter or as described elsewhere herein, amplification may occur on chip (ie in a microwell).

[0064] In certain embodiments, each microwell collects an average of one or fewer cells. In practice, this means that each microwell contains one or zero cells following the method of the present disclosure. In certain embodiments, the collection of one or zero cells per well allows for single cell study. In alternative embodiments, each microwell collects on average more than one cell. Contents of the microwell may then be serially diluted until there is one cell, enabling single cell analysis. For example, by picking up one or more cells and depositing them in another well leading to a serial dilution.

[0065] In certain embodiments, the array of microwells further comprises a microfilter having a porosity that retains fetal trophoblast cells and allows other smaller cell types to pass through. For example, the microfilter may have a porosity in the range of 3 to 40 μm. In certain embodiments, the porosity of the microfilter may range between approximately 1, 3, 5, 7, 8, 10, 12, 15, 16, 20, 25, 30, 35, 40, 45 or 50 μm. In certain embodiments, the pores in the microfilter are round or rod shaped. In certain embodiments, the biological sample is run through the array of microwells at a rate of at least 0.1 mL/min, for example, in a range between 0.5 mL/min and 5 mL/min, or 0.8 mL/min and 4 mL/min or in a range between approximately 0.2 mL/min, 0.6 mL/min, 1 mL/min, 1.5 mL/min, 2 mL/min, 2.5 mL/min, 3 mL/min, 3.5 mL/min, 4 mL/min, 5 mL/min or 10 mL/min. In certain embodiments, the microfilter filters out unwanted smaller cells and retains fetal trophoblastic cells and cellular aggregates of characteristic dimensions larger than that of the filter. In certain embodiments, the microfilter is a microfluidic filter. In certain embodiments, the microfilter is a microfabricated filter membrane. In certain embodiments, the microfilter is located at the bottom of the microarray wells. In certain embodiments, the array of microwells further comprises a microfilter having a porosity that retains fetal trophoblast cells and other cell types to allow removal of excess liquid to concentrate the fetal trophoblast cells and other cell types. For example, the microfilter may have a porosity in the range of 1 to 20 μm. In certain embodiments, the porosity of the microfilter may range between approximately 1, 3, 5, 7, 8, 10, 12, 14, 16, 18 or 20 μm. Accordingly, a liquid volume is reduced and the cells are concentrated without cellular loss that can occur with centrifugation. However, in an embodiment, the microarray wells, do not contain a microfilter, and instead are adapted for the collection of cells (eg using sedimentation).

[0066] In certain embodiments, the microfilter is functionalised with a binding partner that specifically binds to a cellular receptor or specific antigen of trophoblastic cells. In certain embodiments, the cellular receptor or specific antigen may be selected from HLA-G and CD105, although other cellular receptors may be suitable as would be well understood by a person skilled in the art. The binding partner may be a specific antibody or a specific ligand for the cellular receptor or antigen. In certain embodiments, the functionalised microfilter may assist with the specific retention or binding of the fetal trophoblast with the microfilter. Prior to functionalisation, the microfilter may be coated with an agent to reduce background autofluorescence and improve fluorescent imaging of trophoblastic cells. In certain embodiments, the agent comprises at least one of gold, silver, aluminium, platinum or the like. In certain

embodiments, the coating of the agent has a thickness ranging between approximately 10 nm and 300 nm, for example, 20 nm, 25 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 110 nm, 120 nm, 130 nm, 150 nm, 170 nm or 190 nm.

[0067] In certain embodiments, following filtration with the microfilter, more than 98% of the other cell types are removed from the biological sample. In certain embodiments, following filtration with the microfilter, more than 99% of the other cell types are removed from the biological sample. In certain embodiments, following filtration with the microfilter, more than 99.9% of the other cell types are removed from the biological sample. In certain embodiments, following filtration with the microfilter, more than 99.99% of the other cell types are removed from the biological sample. In certain embodiments, following filtration with the microfilter, more than 99.999% of the other cell types are removed from the biological sample.

[0068] In certain embodiments, each of the devices of the method disclosed herein is in microfluidic connection with one or more other devices of the method. In certain embodiments, each of the devices of the method disclosed herein is in microfluidic connection with the other devices of the method. In certain embodiments, the method utilises a combination of microfluidic devices as described herein.

[0069] In certain embodiments, the method further comprises the following step:

(d) staining the collected cells to identify isolated fetal trophoblastic cells.

[0070] The staining may be any type of staining known to persons skilled in the art, providing it identifies fetal trophoblast cells. In certain embodiments, the staining method specifically identifies fetal trophoblasts, for example, the staining may utilise a specific binding partner of a specific cellular receptor or antigen that are understood to be useful in identifying fetal trophoblast cells, such as HLA-G, cytokeratin-7 and/or CD105.

[0071] In certain embodiments, the isolated fetal trophoblastic cells are assessed for genetic or chromosomal abnormalities or placental dysfunctions using standard techniques well understood by persons skilled in the art.

[0072] Accordingly, in an aspect, the present disclosure provides a method of prenatally diagnosing a genetic or chromosomal abnormality or a placental dysfunction in a fetus comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells. In certain embodiments, diagnosis can be performed using cytogenetic, genomic, proteomic, transcriptomic or metabolomics methods and performed using single cells or through pooled analyses of the enriched

fraction. Accordingly, references herein to 'material' may be to, for example, genetic material, proteins or metabolites, depending on the method of analysis or diagnosis. In certain embodiments, the fetal trophoblastic cells are characterised using cytogenetic technology including FISH to identify chromosomal abnormalities. Other suitable diagnostic techniques could also be applied to the cells isolated by the disclosed method as would be understood by the person skilled in the art.

[0073] For example, fetal cell isolation techniques not only have the potential for comprehensive fetal genome analysis, but may allow also simultaneous transcriptome analysis which may enable early biomarker development for pregnancy complications, including preeclampsia, intrauterine growth restriction and stillbirth. In an example, efficient enumeration of circulating fetal cells may also allow risk stratification for these placental complications. Further, the placental transcriptome in pregnancies complicated by preeclampsia differs from healthy pregnancies, which may provide insight into the pathophysiology of disease, allowing development of diagnostic biomarkers and possible therapeutic targets. Trophoblasts and SNA from the placenta circulating in the maternal blood therefore provide a unique opportunity to non-invasively profile placental function in normal and complicated pregnancies. Further, the number of trophoblastic cells increases with pre-eclampsia and so just the number identified provides a prognosis for the development of pre-eclampsia.

[0074] According to an aspect of the present disclosure, there is provided a method for genomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing genetic material from the isolated fetal trophoblast cells. Genomic analysis technologies may include DNA sequencing or array-type assays as would be well understood by a person skilled in the art.

[0075] According to an aspect of the present disclosure, there is provided a method for proteomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells. Proteomic analysis technologies may include affinity arrays or microfluidic separation, such as microchip capillary electrophoresis (or other chromatography- or electrophoresis-based separation) and detection via chemiluminescence or mass spectrometry as would be well understood by a person skilled in the art.

[0076] According to an aspect of the present disclosure, there is provided a method for transcriptomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing genetic material from the isolated fetal trophoblast cells. Transcriptomic

analysis technologies may include RNA sequencing or array-type assays as would be well understood by a person skilled in the art.

[0077] According to an aspect of the present disclosure, there is provided a method for metabolomic analysis of fetal trophoblast cells, the method comprising isolating fetal trophoblast cells from a biological sample according to the method of the present disclosure to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells. Metabolomic analysis technologies may include direct injection mass spectrometry, liquid chromatography coupled to mass spectrometry, gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS), nuclear magnetic resonance spectroscopy, Fourier transform–infrared (FT-IR) spectroscopy and Raman spectroscopy as would be well understood by a person skilled in the art. Also, the person skilled in the art would be understood that mass spectral imaging may use different ionisation methods, for example, matrix assisted laser desorption ionisation (MALDI), desorption electrospray ionisation (DESI) or secondary ion mass spectrometry (SIMS).

[0078] In certain embodiments of the methods disclosed herein, the fetal trophoblastic cells within the microwells are lysed using either a micromanipulator or manual pipetting. In certain embodiments, a lysis buffer is delivered to individual microwell to lyse the cells of interest. The cell lysate can then be aspirated for genetic analysis. In certain embodiments, the RNA or DNA from the lysed trophoblastic cells is amplified *in situ* in the microwells (eg in microwells, in water droplets in oil or on the filter). In alternative embodiments, amplification of the DNA or RNA can occur following removal from the microwells. In some embodiments, lysed trophoblastic cells are characterized using genomic transcriptomic, proteomic and/or metabolomic technologies as described herein and as would be well understood by a person skilled in the art.

[0079] In an aspect, the present disclosure provides an apparatus comprising a combination of microfluidic devices as described herein. In certain embodiments, the apparatus mediates the isolation of fetal trophoblastic cells and/or SNAs from a suitable biological sample.

EXAMPLES

[0080] EXAMPLE 1: DESIGN AND FABRICATION OF INERTIAL MICROFLUIDIC DEVICES

[0081] Various designs of inertial microfluidic device were used or developed for enrichment of trophoblastic cells. A first inertial microfluidic device ('device 1') comprised 2 inlets and 2 outlets having a channel that had a width of 500 μm and a height of 160 μm with a total path length of about 100 mm. The device was fabricated with photolithography and comprised PDMS bonded to a glass slide or PDMS

bonded to PDMS. A second inertial microfluidic device ('device 2') comprised an 8-loop spiral microchannel with one inlet and two outlets with the radius increasing from 8 mm to 24 mm. The width of the channel cross-section was 600 μm, and the inner/outer heights were 80 and 130 μm, respectively, for the trapezoid cross-section. The device was fabricated through micromilling and soft lithography and comprised PDMS bonded to PDMS. Both devices are effective for enrichment of trophoblastic cells.

[0082] EXAMPLES 1A AND 1B: ENRICHMENT OF TROPHOBLASTIC CELLS

[0083] Example 1A

[0084] Calcein stained JEG3 cells (a placental cell line) spiked into whole blood were enriched using an inertial microfluidic device as described herein (device 2) (See, also, 'Enrichment step 1' in Figure 1) and recovery was enumerated with imaging flow cytometry (Figure 2). Specifically, JEG3 cancer cells were spiked into 8-9 mL of whole blood (ie 'pre-sample') with <1000 JEG3 cells per mL of blood and the red blood cells were lysed leaving white blood cells (WBC) and the JEG3 cells intact (ie nucleated cells). Nucleated cells were resuspended in PBS at 3x initial blood volume, but this can vary according to the number of cells recovered. The nucleated cell fraction was pushed through the inertial microfluidic device with a syringe pump at 1700 μL/min, (but this can change with device optimisation) and sample was collected from the trophoblast outlet (ie large cell outlet of the inertial microfluidic device).

[0085] There was greater than 99.9% WBC depletion with the inertial microfluidic device (approximately 2000-6,000 WBC contaminate per mL of blood). That is, after a single pass through the inertial microfluidic device approximately 2-6 million WBC per mL of blood was reduced to 2-6,000 WBC per mL of blood. The initial WBC number was enumerated with a flow cytometer and used to calculate depletion.

[0086] After a second pass through the inertial microfluidic device, the number of WBC was reduced to less than 1000 contaminating WBC per mL of blood. That is, approximately 90% depletion from the previously separated sample. Approximately 80% of the JEG3 cells (~6000 cells, from 8000 JEG3 cells spiked into 8 mL of fresh blood) were recovered after a single pass through the inertial microfluidic device. Approximately 54% of JEG3 cells were recovered following two passes through the inertial microfluidic device.

[0087] Figure 2 shows an example of a typical scatter plot of calcien intensity against brightfield (BF) cellular area from (A) a pre-sample and (B) a sample obtained from the trophoblast outlet of the inertial microfluidic device. (C) is an image of JEG3 stained cells, and (D) is an image of unstained white blood cells. Images taken at 400x magnification and have been adjusted to enhance visual appearance.

[0088] Example 1B

[0089] Fetal trophoblastic cells from a pregnant woman's blood sample were enriched using an inertial microfluidic device as described herein (device 2). Maternal blood was collected in two 10mL EDTA tubes with 16mL of blood used in the procedure. Maternal blood was kept on ice or at 4°C and processed within 3 hours after collection. Blood was taken off ice and left to come to room temperature for ~15 minutes before processing. 16mL of blood was added to 64mL of RBC lysis buffer (gBioscience) and incubated at room temperature on a shaker for 15 minutes. Sample was then centrifuged with soft deceleration at 500g for 10 minutes. Supernatant was removed and discarded. The white blood cell pellet was first resuspended in 1mL of PBS (tips pre-coated in Pluronic to reduce binding), an additional 47mL of PBS was then added. Sample was then loaded into a syringe, placed in a syringe pump and connected to the inertial microfluidic device. Sample was run through the inertial microfluidic device at 1700µL/minute and the trophoblastic (large cell) outlet was collected and rerun through the spiral (double pass). The trophoblastic outlet was then centrifuged at 500g for 10 minutes, supernatant removed and the cell pellet resuspended in 100µL PBS with 1% fetal bovine serum. HLA-G APC (0.5ug/100µL) and CD45 FITC (0.5µL/100µL) fluorescent antibodies were then added and incubated for 30 minutes on ice. After 20 minutes Hoechst was added and incubated for 10 minutes. After incubations PBS was added and the sample centrifuged. Supernatant was removed and the sample was washed in PBS. Using the 5000µm well (in a 35mm hydrophobic petri dish) with an array of 4 wells, cells were centrifuged (500 g for 10 minutes) to concentrate. Then 2 mL of PBS was added to the petri dish with microwells and 1 mL of mineral oil is added on top of media. Using a pipette 1850 µL of media was removed from the petri dish below the oil leaving behind a thin media layer. The concentrated cells in ~20 µL (maximum 10,000 per well fresh or fixed) were then added to this thin layer (~5µL per well), which restricts movement of the cells. The well was then automatically scanned with a fluorescence microscope (Nikon TiE) and the cell of interest identified (CD45 negative, Hoechst positive with or without HLA-G).

[0090] Figure 3 shows micrographs of a HLA-G and Hoechst positive, CD45 negative JEG3 cell (square box) from after separation using an inertial microfluidic device as described herein (device 2), images taken at 100× magnification. A) Hoechst, B) HLA-G, C) CD45, and D) brightfield (taken at 400 × magnification).

[0091] EXAMPLE 2: OPTIMISATION OF FLOW RATES AND PORE SIZES FOR FILTRATION WITH MICROFILTERS

[0092] The recovery of trophoblastic cells from a trophoblastic cell line was analysed using different flow rates and filter pore sizes of microfilters in a microarray, as shown in Table 1. Specifically, sample from a single pass through the inertial microfluidic device as described herein (device 2) was passed through different SU8 microfilters using a syringe pump. Filters with round pore sizes of 12 µm

and 16 µm were used. The flow rates of 1mL/min, 1.5 mL/min, 2 mL/min and 2.5 mL/min were used. The results show that 99.55 % WBC depletion was obtained at a flow rate of 2.5 mL/min using a 16 µm pore size, which was the optimum for depletion. Calculation of % WBC depletion is based on how many WBC are retained on the filter and the number before filtration, eg, a low number of WBC on the filter will produce a high WBC depletion %.

[0093] **Table 1**: Recovery of Fetal trophoblastic cells (FTCs) using micro fabricated filters AT different flow rates and round pore sizes.

Flow Rate mL/min	Pore Size (µm)	% WBC depletion
1	12	93.52
	16	97.01
1.5	12	97.64
	16	97.80
2	12	98.41
	16	98.48
2.5	12	98.44
	16	99.55

[0094] EXAMPLE 3: RECOVERY OF TROPHOBLASTIC CELLS USING DIFFERENT MICROFILTERS

[0095] Using the methods described herein, the recovery of trophoblastic cells from a trophoblastic cell line was analysed using different microfilters in a microarray, as shown in Table 2. Specifically, sample from a single pass through the inertial microfluidic device as described herein (device 2) was passed through the SU8 microfilter using a syringe pump. Four different SU8 filters were used with 6, 8, 12 and 16 µm rod shaped pores. The flow rate was varied from 250µL/min to 5mL/min. The results show that the highest recovery was obtained using a filter with 6 µm rod pores.

[0096] **Table 2**: Recovery of Fetal trophoblastic cells (FTCs) using micro fabricated filters with rod shaped pores.

Pore Size	Microwell Design	Flow Rate	Target Cell recovery
6μm	Square	500μL/min	97.6%
8μm	Square	500μL/min	97.3%
8μm	Honeycomb	1mL/min	85.5%
12μm	Square	1mL/min	84.7%
12μm	Square	2.5mL/min	79.2%
12μm	Square	5mL/min	22.8%
12μm	Honeycomb	250μL/min	87.8%
12μm	Honeycomb	500μL/min	85.9%
12μm	Honeycomb	1mL/min	84%
12μm	Honeycomb	2mL/min	83.8%
16μm	Square	500μL/min	61.3%
16μm	Square	750μL/min	47.9%

[0097] EXAMPLE 4: RECOVERY OF TROPHOBLASTIC CELLS USING FUNCTIONALISED MICROFILTERS

[0098] The recovery of trophoblastic cells from a trophoblastic cell line was analysed using functionalised and non-functionalised microfilters in a microarray, as shown in Table 3. Microfilters functionalised with 11-Mercaptoundecanoic acid (MUA) or polyethylene glycol (PEG) were prepared by coating the standard SU8 filters in a 25-100nm gold layer (See Figure 4 for a photo of gold 150μm wells (A) and 500μm wells (B) taken before functionalisation). Filters were washed with 100% ethanol and surface cleaned with oxygen plasma. For MUA functionalisation, the filters were soaked in MUA (alkane acid and alkane thiol) solution overnight, washed with ethanol, then MQ water and then activated with N-(3-dimethyl amino propyl)-N-ethylcarbodiimide hydrochloride (EDC) for 10 minutes. For PEG functionalisation, the filters were soaked in PEG (HS-PEG-COOH and HS-PEG-OME) solution overnight, washed with MQ water and then activated with N-hydroxysuccinimide (NHS) for 10 minutes. Filters were washed with MQ water, then PBS, and then conjugated with HLA-G antibodies for 2 hours. Filters were washed with 1×PBS, blocked with bovine serum albumin (BSA) 5%, and then washed with PBS. The sample was run through the filters as described herein. A flow rate of 1mL/min was used as a standard.

[0099] As expected there is a greater recovery with the 12µm pore size (as fewer cells are able to easily pass through), but there is also a greater recovery of JEG3 cells with the functionalised filter compared to the non-functionalised. The 16µm pore filter also shows a greater recovery of JEG3 cells in the functionalised vs not-functionalised demonstrating the improved specific recovery that functionalisation can achieve.

[00100] **Table 3**: Recovery of Fetal trophoblastic cells (FTCs) using gold coated micro fabricated filters (Square microwells, 1ml/min flow rate).

Rod Size	**Surface**	**Recovery**
12µm	Not-Functionalised	77%
12µm	Functionalised	89%
16µm	Not-Functionalised	74%
16µm	Functionalised	87%

[00101] EXAMPLE 5: STAINING OF TROPHOBLASTIC CELLS ON MICROFILTER

[00102] After separation with the inertial microfluidic device as described herein (device 2) and filtration, cells were stained on the filter with specific antibodies to distinguish WBC from fetal trophoblastic cells. This was done by passing, for example, antibodies/fixative/ solutions through the filter as per sample from the inertial microfluidic device. Cells were stained with HLA-G (either fresh or fixed) and Hoechst (fresh), or Cytokeratin (fixed) and DAPI (fixed) to specially stain trophoblastic cells. Cells were fixed with formalin, glutaraldehyde or ice cold methanol (and permeabilized with Triton-x 100). Cells were processed (stained/fixed permeabilized) while still in a holder on the syringe pump at a flow rate of 100 µL/min and then the flow was stopped to reduce the amount of antibody used. The cells were visualised using fluorescence and brightfield microscopy, with trophoblastic cells distinguished based on staining and their morphology (ie trophoblastic cells were larger than the surrounding WBC).

[00103] Figure 5 shows a single stained JEG3 (primary HLA-G antibody with secondary Anti-mouse IgG FITC antibody and Hoechst) in 500 µm well (A) and stained JEG3 (Cytokeratin FITC +DAPI) in 220 µm honeycomb well (B). Detection of HLA-G or Cytokeratin and a nuclear signal indicates the presence of a fetal cell, the cell can then be picked up or lysed with a manual pipette or single cell manipulator system.

[00104] EXAMPLE 6: SINGLE CELL PICK UP FOR DOWNSTREAM ANALYSIS

[00105] Fetal cells were isolated from maternal blood by enrichment with an inertial microfluidic device as described herein (device 2), collected into a microarray as described herein and single cells were picked-up as described below.

[00106] Method 1 (microwell only):

[00107] Using the 5000µm well (in a 35mm hydrophobic petri dish) with an array of 4 wells, cells were centrifuged (500 g for 10 minutes) to concentrate. Then 2 mL of cell culture media/PBS was added to the petri dish with microwells and 1 mL of mineral oil is added on top of media. Using a pipette 1850 µL of media was removed from the petri dish below the oil leaving behind a thin media layer. The concentrated cells in ~20 µL (maximum 10,000 per well fresh or fixed) were then added to this thin layer (~5µL per well), which restricts movement of the cells making pick up easier.

[00108] The microwell was either manually or automatically scanned with a fluorescence microscope detected by setting fluorescent thresholds. i.e. pick up CD45 negative cytokeratin/HLA-G positive. Cells of interest were identified based on their brightfield, nuclear staining and/or specific antibody stain (i.e. HLA-G or Cytokeratin) and either manually or automatically chosen on the computer, with detection based upon size and also presence of positive/negative staining. Cellsorter software (https://www.singlecellpicker.com/) was used for this which is an automated single cell manipulator, but can also use a number of other proprietary software. The Cellsorter software provided for automated detection, based on user defined parameters, cell minimum area, cell maximum area, detection sensitivity, cell minimum intensity, cell maximum intensity, noise threshold and comparison of these values with other channels.

[00109] Single cells were either manually or automatically picked up using a programmable motorised arm attached to a micropipette (normally 30-70µm inner diameters) and deposited into a PCR tube, glass cover slip or into another microwell (for serial dilution) or water in oil droplet. To achieve this, a small vacuum was applied to the micropipette (commercially available single cell manipulation technology). Once single cells were isolated they were used for various downstream applications, such as Karyomapping, Next Generation Sequencing, fluorescence *in situ* hybridisation and droplet digital PCR.

[00110] Figure 6 shows JEG3 cells within a microwell (in a thin layer of media with mineral oil on top to facilitate for pick up) before (A) and after pick up with a 30µm needle (B). In this instance 1 cell was picked up and deposited into a PCR tube as shown this can easily enable downstream analysis techniques (C); images taken at 100× magnification. Cells can also be deposited onto a glass slide (D);

image taken at 400× magnification. The positive deposition of a single cell can be visualised with fluorescence microscopy.

[00111] Figure 7 shows a cytokeratin positive (green) DAPI positive cytotrophoblast cell isolated from a pregnant woman's blood sample before (A) and after pick up (B). Sample was stained for Cytokeratin, DAPI and CD45. Image taken at 400× magnification.

[00112] Method 2 (filter or microwell):

[00113] Cells were filtered (see previous section for details), stained (fresh or fixed; see previous section for details) and then the filter was placed with PBS into a specially designed holder, so that the filter did not touch the bottom of the petri dish and liquid did not pass through.

[00114] Cells were found in the same way as Method 1, and were picked up with the micropipette as an 'adherent' cell (same method but slightly increased pressure) which provides greater control over the process than suspension cells as the cells are stationary.

[00115] Depending on the previous enrichment and use of microfilter, there were 1 or fewer cells per microwell. Sample from a single pass through the inertial microfluidic device was passed through a SU8 microfilter using a syringe pump. Samples can be fixed and then stained with Cytokeratin/ HLA-G, CD45 and DAPI if required. Filters are then removed from the holder and are imaged with fluorescence microscopy. Once a cell of interest has been identified it can either be picked up by the single cell manipulator, or lysed on filter or in a water in oil droplet, with subsequent removal of DNA/RNA for downstream genetic analysis.

[00116] Figure 8 shows a micrograph of a microfilter with 500μm wells (brightfield) and an 80μm single cell manipulation micropipette (red circle) able to pick up cells within the well, and dispense/remove lysis buffer/lysed solution respectively.

[00117] EXAMPLE 7: SINGLE CELL LYSIS FOR DOWNSTREAM ANALYSIS

[00118] After sample recovery from the inertial microfluidics device as described herein (device 2) and filtration with the microwell/filter system with or without staining, a cell or cells within an individual microwell or water in oil droplet underwent cellular lysis. A standard cell lysis buffer can be used (eg 10mM Tris-CL, 100mM NaCL, 1mM EDTA, 1% TritonX-100, 10% glycerol, 1%SDS and 0.5% sodium deoxycholate or 400mM KOH, 10mM EDTA, 100mM DTT) or propriety buffers as used on laser capture microdissection or for example Singleshot cell lysis kit – BioRad or Single Cell Lysis Buffer for Whole Genome Amplification DOPlify™- Reproductive Health Science). A manual or automated pipette was

used to dispense the lysis solution (volume dependent on the size of the microwell), which was left on top of the cell for ~15 minutes. The solution containing the lysed cell was then picked up in the same way it was deposited and then deposited into a PCR tube for further downstream analysis.

[00119] Having 1 cell/well allows us to lyse 1 single cell for further analysis (i.e. droplet digital PCR or Next Generation Sequencing). Having small wells (150µm) allows for cellular contents to be contained within a small volume, making downstream analysis easier. Advantageously, having a cell in small (eg. 10, 15, 20 or 25 micron) water in oil droplet making downstream analysis even easier.

[00120] Figure 9 shows a single JEG3 cell (circle) lysis within the microwell, before (A) and after lysis (B) of a single cell within a 500 µm microwell that is removed. (C) Visual representation demonstrating single well buffer manipulation (in this case red dye), in order to specifically target and lyse 1 microwell.

[00121] Figure 10 shows a single stained cell (Square) before (A) and after (B) lysis in a 500µm honeycomb microwell. An example of a droplet digital PCR profile for KRT19 (Keratin 19), in this instance multiple cells were lysed on filter, the mRNA was recovered, converted to cDNA before undergoing PCR (QX200™ droplet digital PCR system BioRad). The droplet digital PCR plot shows positive droplets i.e. the presence of KRT19 mRNA after lysis of multiple cells. This demonstrates that the genetic material can be recovered after lysing the cells.

[00122] EXAMPLE 8: ENRICHMENT AND COLLECTION OF FETAL CELLS FROM MATERNAL BLOOD

[00123] Fetal cells were isolated from maternal blood by enrichment with an inertial microfluidics device as described herein (device 2) and collected into a microarray as described herein. Enrichment and collection were performed as per Example 1B with the following variations. Maternal blood was collected in two 10mL EDTA tubes (or Streck BCT® cell free DNA tubes, kept at room temperature and processed within 24 hours).

[00124] Alternatively, fetal cells were isolated from maternal blood as follows: 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 (proprietary lysis buffer or dextran could also be used). After 10 minutes of vigorous shaking, samples were centrifuged at 500g for 10 minutes, the cell pellet was then washed and resuspended in PBS (eg. up to ×6 dilution) and the cellular suspension was loaded in a syringe.

[00125] Optionally, the sample may be fixed before red blood cell lysis and before running through the inertial microfluidic device. Samples were fixed with 4% formaldehyde for 10 minutes at room temperature, red blood cell were then lysed using 0.1% Triton X 100 for 10 minutes at room temperature in PBS. Sample is then centrifuged for 10 minutes at 500g to recover the nucleated cell fraction, supernatant is removed and sample is resuspended in PBS.

[00126] From this point an inertial microfluidics device as described herein (device 2) was used to further enrich the fetal cells obtained from the maternal sample. The suspension was injected through tygon tubing with a syringe pump into the inertial microfluidic device (1700 μL/min) using a standard syringe pump. Eluted suspension, enriched in trophoblastic cells, were then collected and processed for downstream analyses. In one instance, sample from the trophoblast outlet was plated into well plates, the samples were left to adhere for approximately 24 hours and then stained with May-Grünwald Giemsa. Briefly, May-Grünwald dye was placed onto the plated cells and incubated for 5 minutes. Giemsa dye was diluted 1:20 with autoclaved MQ water, added to the cells and incubated for 20 minutes. The sample was washed with water and left to air dry. Microscopy was carried out using a Nikon Ti Eclipse inverted Microscope fitted with a colour camera. In another instance, sample from the trophoblastic outlet was analysed using an imaging flow cytometer (IFC). The cells were stained with the nuclear stain DAPI (4',6-diamidino-2-phenylindole) and with the fluorescently labelled fluorochromes Anti-Human CD45 PE-Cy5.5 and Anti-Pan-Cytokeratin (AE1/AE3) Alexa Fluor® 488 (eBioscience, San Diego, CA, USA). IFC 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 400× magnification and 10,000 – 500,000 events were collected depending on sample volume.

[00127] The results are in Figure 11 which shows (A) a micrograph of multinucleated syncytial nuclear aggregate (SNA) stained with May- Grünwald Giemsa (400x magnification), and (B) SNAs identified as being keratin positive (pan-KRT) and CD45 negative by imaging flow cytometry (400x magnification).

[00128] EXAMPLE 9: ISOLATION OF SYNCYTIAL NUCLEAR AGGREGATE FROM MATERNAL BLOOD WITH PRE-ECLAMPSIA

[00129] A syncytial nuclear aggregate was isolated from the blood of a pregnant woman with pre-eclampsia using the enrichment step as described herein, with an inertial microfluidic device as described herein (device 2). Enrichment and collection were performed as per Example 1B with the following variations. Maternal blood was collected in two 10mL EDTA tubes (but could also be collected in Streck BCT® cell free DNA tubes, kept at room temperature and processed within 24 hours).

[00130] The results are in Figure 12 which shows micrographs of a CD45 negative, Hoechst Positive syncytial nuclear aggregate from a pregnant women's blood sample (pregnancy complicated with pre-eclampsia) after separation with images A, B, C taken at 400× magnification. A) Brightfield before picking, B) Hoechst before picking, C) CD45 FITC before picking, and D) Hoechst after picking demonstrating efficient pick up of the designated cell (taken at 100 × magnification).

[00131] EXAMPLE 10: ISOLATION OF FETAL CELLS FROM MATERNAL BLOOD WITH CONFIRMED TRISOMY 21

[00132] Fetal cells with confirmed trisomy 21 were isolated from maternal blood using the enrichment step as described herein, with an inertial microfluidic device as described herein (device 2). 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 vigorous 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 an inertial microfluidic device was used to further enrich the fetal cells obtained from the maternal sample. The suspension was injected through tygon tubing with a syringe pump into the inertial microfluidic chips (1700 µL/min) using a standard syringe pump. Eluted suspension, enriched in trophoblastic cells, were then collected and processed for downstream analyses for FISH and IFC. Sample from the trophoblastic outlet analysed using an IFC. The cells were stained with the nuclear stain DAPI (4',6-diamidino-2-phenylindole) and with the fluorescently labelled fluorochromes Anti-Human CD45 PE-Cy5.5 (0.5µg/100µL) and Anti-Pan-Cytokeratin (AE1/AE3) Alexa Fluor® 488 (0.25µg/100µL) (eBioscience, San Diego, CA, USA). IFC 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. Events per sample were acquired at 400× magnification and 10,000 – 500,000 events were collected depending on sample volume. For FISH, maternal blood samples were processed and the large cells were isolated with an inertial microfluidics as described above. 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°C in Carnoy's fixative until further processing. 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 (either probe set X spectrum green, Y spectrum orange, 18 spectrum aqua or 13 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 hybridised at

75°C for 5 minutes, before being incubated at 30°C overnight. Slides were washed with 0.4× Saline-sodium citrate buffer (SSC) with NP-40 (pH 7) at 75°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 a Nikon Ti Eclipse inverted fluorescence microscope and the CompuCyte iCys™ Laser Scanning Cytometer using DAPI , FITC (*) and Cy3/PE (+) channels for detection of nuclei, Chromosome X (or 13 in Trisomy 21 (T21) sample) and chromosome Y (or 21 in T21 sample), respectively.

[00133] The results are shown in Figure 13 which shows (A, C) an example of a computed colour image of a cytotrophoblast with fluorescence *in situ* hybridisation probing for chromosomes 13 (*) and 21 (+); and (B, D) Raw red imaging of chromosome 21 signals in the same cell as A and C, respectively. A cell from a trisomy 21 pregnancy has also been isolated, amplified and sequenced demonstrating the applicability of these samples for more complex downstream analysis.

[00134] EXAMPLE 11: ISOLATION OF FETAL CELLS FROM MATERNAL BLOOD WITH MALE FETUSES

[00135] The protocol described in Example 10 was repeated for pregnancies with a male fetus. Fetal cells from maternal blood were enriched using the enrichment step as described herein, with an inertial microfluidic device as described herein (device 2). Blood samples were collected in Streck BCT® cell free DNA tubes, kept at room temperature and processed within 24 hours. Whole blood was fixed with 4% formaldehyde for 10 minutes at room temperature. Red blood cells were then lysed with 0.1% Triton X 100 in PBS after 10 minutes of vigorous shaking, samples were centrifuged at 500g for 10 minutes, the cell pellet was then washed and resuspended in PBS (×6 dilution) and the cellular suspension was loaded in a syringe. From this point an inertial microfluidic device was used to further enrich the fetal cells obtained from the maternal sample. The suspension was injected through tygon tubing with a syringe pump into the inertial microfluidic chips (1700 µL/min) using a standard syringe pump. Eluted suspension, enriched in trophoblastic cells, were then collected and processed for downstream analyses for FISH and/or immunofluorescence (IF) in a microwell before then performing FISH. The cells were stained with the nuclear stain DAPI (4',6-diamidino-2-phenylindole) and with the fluorescently labelled fluorochromes Anti-Human CD45 APC and Anti-Cytokeratin FITC (Miltenyi Biotec). After staining cells were placed in a microwell and scanned as previously described. Cytokeratin positive cells were collected using the single cell manipulator before being deposited onto a glass slide for FISH (in one instance). For FISH, maternal blood samples were processed and the large cells were isolated with an inertial microfluidics as described above. The trophoblast outlet sample was deposited onto a glass slide and then treated with hypotonic solution (0.05mol/L potassium chloride) and left at room temperature for 10 minutes before removal. Carnoy's fixative (3:1 methanol/acetic acid) was then added to the slide and removed three times. Slides were left to air dry before probes (either probe set X spectrum green, Y

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 hybridised at 75°C for 5 minutes, before being incubated at 30°C overnight. Slides were washed with 0.4× Saline-sodium citrate buffer (SSC) with NP-40 (pH 7) at 75°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 a Nikon Ti Eclipse inverted fluorescence microscope using DAPI, FITC and Cy3 channels for detection of nuclei, Chromosome X and chromosome Y, respectively. In this instance we recover 0.6 circulating fetal cells per mL of mother's blood.

[00136] Figure 14 shows examples of fluorescence *in situ* hybridisation of circulating fetal cells from 3 pregnancies with male fetuses enriched with inertial microfluidics (DAPI nuclear stain, Y chromosome: + (DYZ3), X chromosome: * (DXZ1)). Images taken at 400× magnification.

[00137] Figure 15 shows an example of an isolated circulating fetal cell which after enrichment was stained with specific markers and DAPI nuclear staining (A) and placed in a microwell. This cell was then recovered and placed onto a slide for fluorescence in situ hybridisation for X (*) and Y (+) chromosomes confirming that this fetal cell was from a male fetus (B).

[00138] EXAMPLE 12: USE OF WATER IN OIL DROPLETS AS MICROWELLS

[00139] In these examples, the step of collecting isolated fetal trophoblast cells was carried out by applying the enriched fetal trophoblast cell population or the purified fetal trophoblast cell population to water droplets in oil. The water droplets behave in the same way as microwells allowing containment of the cell or lysis/amplification within the droplet.

[00140] Figure 16 shows an example of a water (represented by #) in oil (represented by *) droplet containing a single cell (circle), Brightfield image (A) and nuclear DAPI image (B) of the same droplet attached to a glass surface. An image of a microneedle (arrow) depositing a water droplet (C) and of a water droplet containing multiple cells which was free floating in the oil droplet (D).

[00141] Droplet size can vary from microlitres to nanolitres and the water droplet acts as a reaction vessel to contain cells for further analysis including lysis and genomic amplification. Each oil droplet may contain 1 or more water droplets.

[00142] Droplets may contain 1 or more cells and may be free floating, or attached to the surface. The surface chemistry of the glass may be altered (to change contact angle) so that the water droplet forms a near perfect sphere while also being attached to the surface.

[00143] Advantageously, DNA/RNA amplification may occur within the droplet, which may be located, for example, on a filter or on chip (ie in a microwell).

[00144] As is apparent from the present disclosure, the methods of the present disclosure provide isolated fetal cells that can potentially be used for non-invasive prenatal diagnosis using cytogenetic, genomic, proteomic or transcriptomic methods.

[00145] Throughout the specification and the claims that follow, unless the context requires otherwise, the words "comprise" and "include" and variations such as "comprising" and "including" will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers.

[00146] The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement of any form of suggestion that such prior art forms part of the common general knowledge.

[00147] It will be appreciated by those skilled in the art that the disclosure is not restricted in its use to the particular application described. Neither is the present disclosure restricted in its preferred embodiment with regard to the particular elements and/or features described or depicted herein. It will be appreciated that the disclosure is not limited to the embodiment or embodiments disclosed, but is capable of numerous rearrangements, modifications and substitutions without departing from the scope of the disclosure as set forth and defined by the following claims.

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CLAIMS

1. A method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b.i) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population;

(b.ii) optionally purifying the enriched fetal trophoblast cell population by applying the enriched fetal trophoblast cell population to a deterministic lateral displacement (DLD) device to obtain a purified fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying a cell population selected from the enriched fetal trophoblast cell population or the purified fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

2. A method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b.i) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device optimised to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population;

(b.ii) purifying the enriched fetal trophoblast cell population by applying the enriched fetal trophoblast cell population to a deterministic lateral displacement (DLD) device to obtain a purified fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying the purified fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

3. A method of isolating fetal trophoblastic cells from a suitable biological sample comprising the following steps:

(a) providing a suitable biological sample comprising fetal trophoblastic cell population within a population of other cell types;

(b) enriching the fetal trophoblastic cell population by applying the biological sample to an inertial microfluidic device to separate fetal trophoblastic cells from the population of other cell types to obtain an enriched fetal trophoblast cell population; and

(c) collecting isolated fetal trophoblast cells by applying the enriched fetal trophoblast cell population to an array of microwells to collect isolated fetal trophoblast cells.

4. The method of any one of claims 1 to 3 wherein the step of enriching the fetal trophoblastic cell population is performed two, three or more times to further enrich the fetal trophoblast cell population.

5. The method of any one of claims 1 to 4 wherein the suitable biological sample is a maternal biological sample.

6. The method of any one of claims 1 to 4 wherein the suitable biological sample is selected from maternal blood, maternal cervical mucus, maternal endocervical canal, maternal endometrial cavity, from lavage of the maternal endocervical canal or the uterine cavity, and endometrial biopsy.

7. The method of any one of claims 1 to 6 wherein, following step (c), each microwell within the array of microwells collects an average of one or fewer cells.

8. The method of any one of claims 1 to 6 wherein, following step (c), each microwell within the array of microwells collects an average of one or more cells.

9. The method of any one of claims 1 to 8 wherein the array of microwells further comprises a microfilter having a porosity that retains fetal trophoblast cells and allows other cell types to pass through.

10. The method of any one of claims 1 to 8 wherein the array of microwells further comprises a microfilter having a porosity that retains fetal trophoblast cells and other cell types to allow removal of excess liquid to concentrate the fetal trophoblast cells and other cell types.

11. The method of claim 9 or 10 wherein the microfilter is coated with an agent to reduce background autofluorescence and improve fluorescent imaging of fetal trophoblast cells.

12. The method of any one of claims 9 to 11 wherein the microfilter is functionalised with a binding partner that specifically binds to cellular receptors of trophoblastic cells.

13. The method of any one of claims 1 to 12 wherein the method further comprises the following step:

(d) staining the collected cells to identify isolated fetal trophoblastic cells.

14. The method of any one of claims 1 to 13 wherein the isolated fetal trophoblastic cells are assessed for genetic or chromosomal abnormalities or placental dysfunctions.

15. A method of prenatally diagnosing a genetic or chromosomal abnormality or a placental dysfunction in a fetus comprising isolating fetal trophoblast cells from a biological sample according to the method of

any one of claims 1 to 14 to obtain isolated fetal trophoblast cells and assessing material from the isolated fetal trophoblast cells.

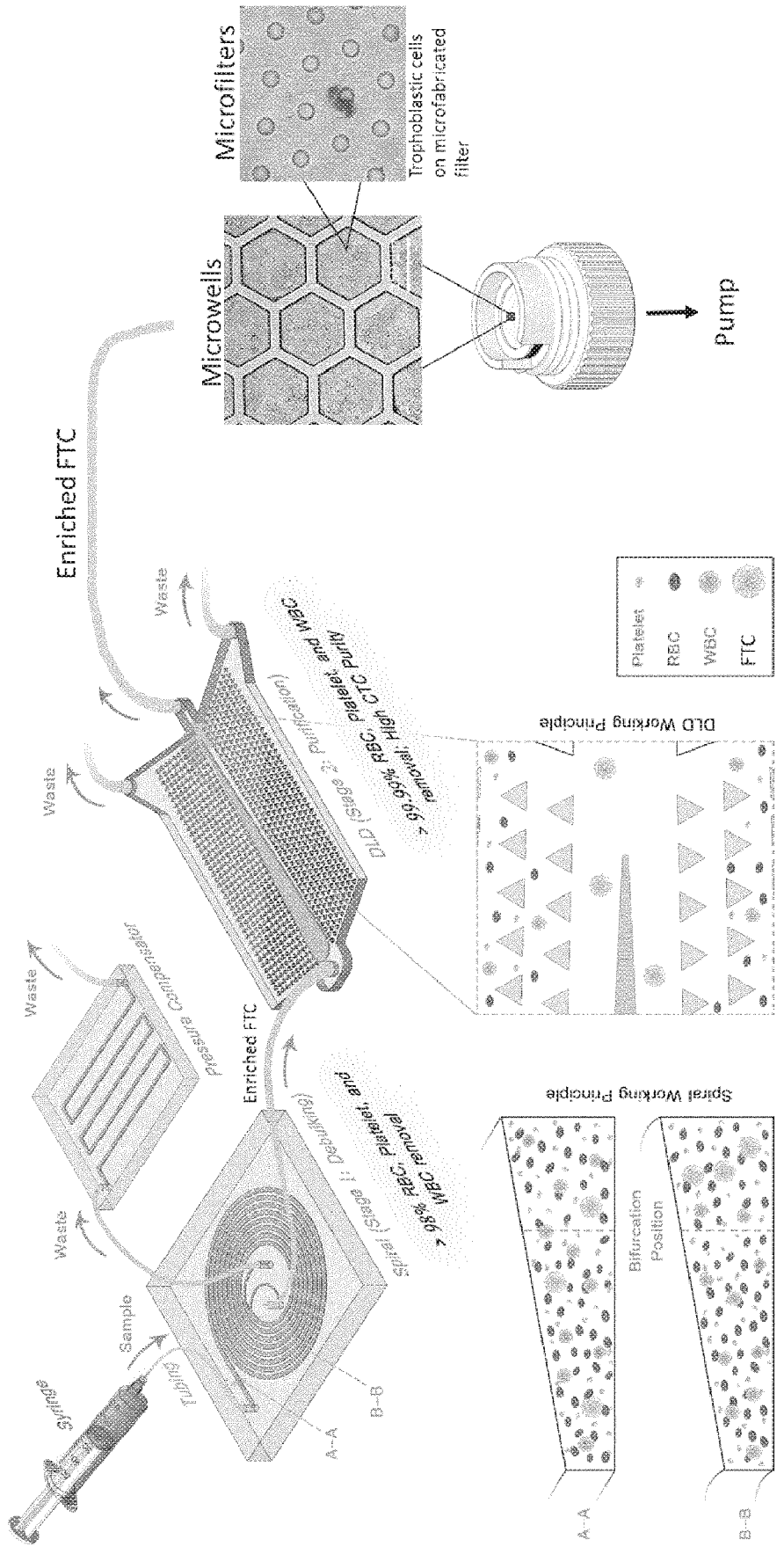
Enrichment step 1
Enrichment step 2 (Optional)
Enrichment/collection step 3
Syringe
Tubing
Sample
Waste
Pressure Compensator
Spiral (Stage 1: Debulking)
Spiral: Platelet, and >98% RBC, WBC removal
Enriched FTC
DLD (Stage 2: Purification)
DLD: >99.99% RBC, platelet, and WBC removal; High CTC purity
Enriched FTC
Microwells
Microfilters
Trophoblastic cells on microfabricated filter
Pump
Spiral Working Principle
Bifurcation Position
A-A
B-B
DLD Working Principle
Platelet
RBC
WBC
FTC

Figure 1

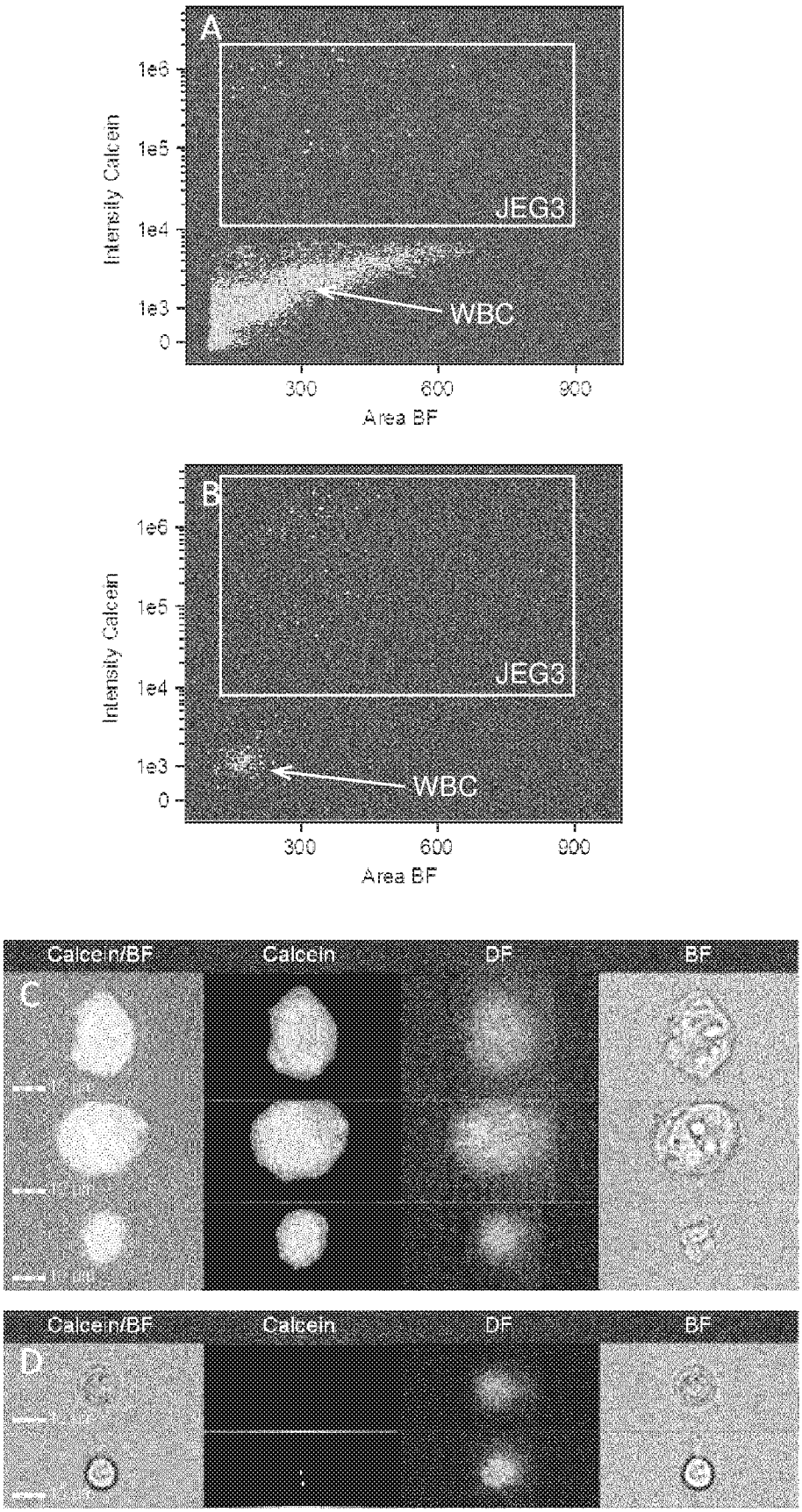

Figure 2

SUBSTITUTE SHEET (RULE 26)

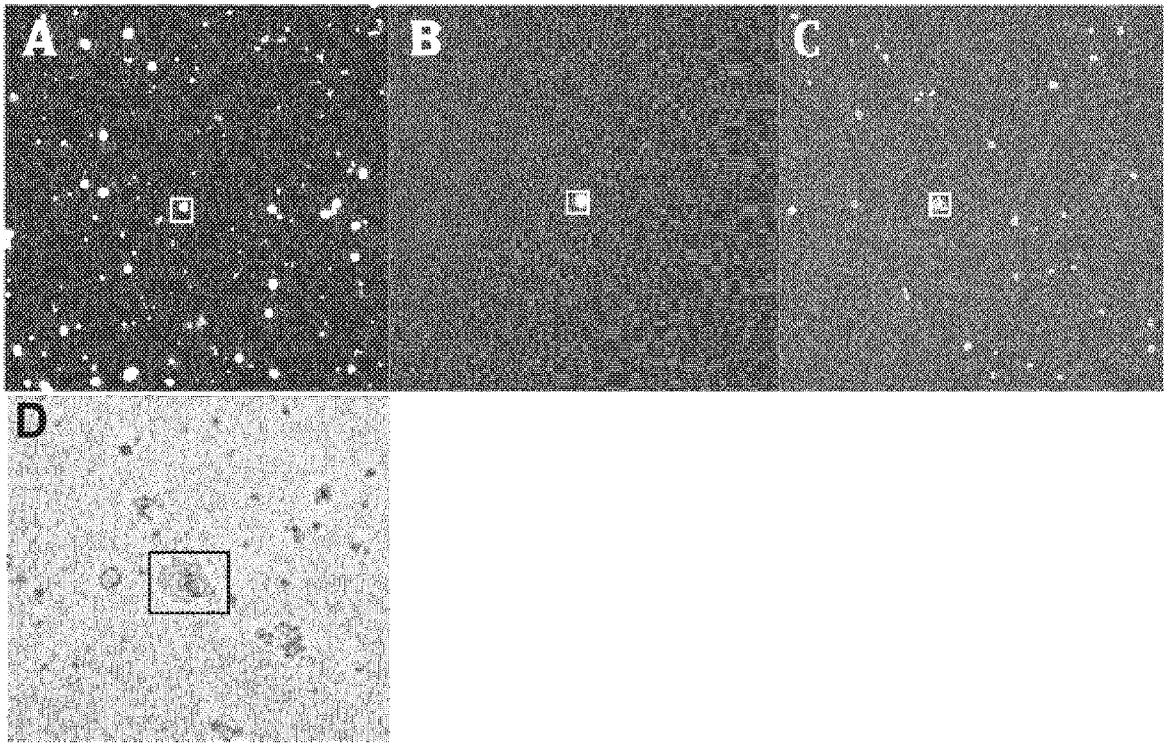

Figure 3

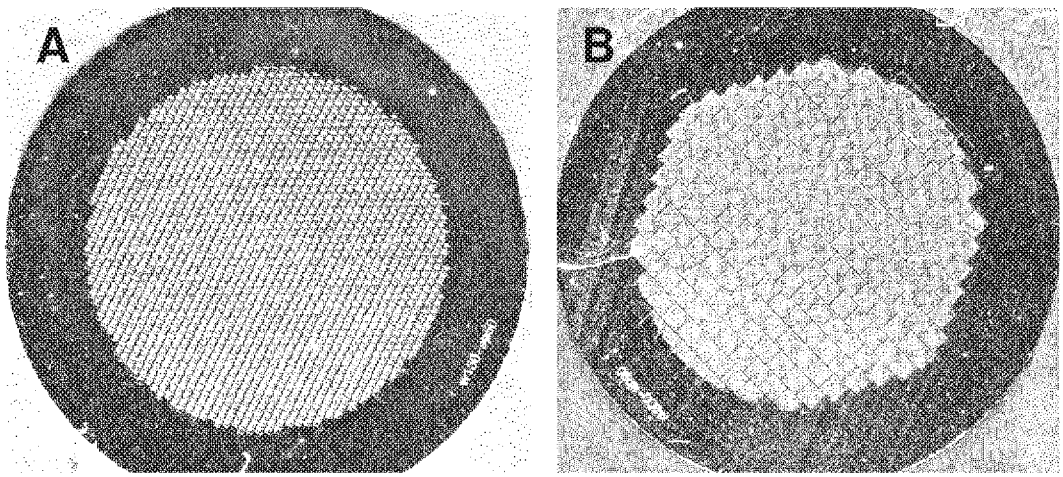

Figure 4

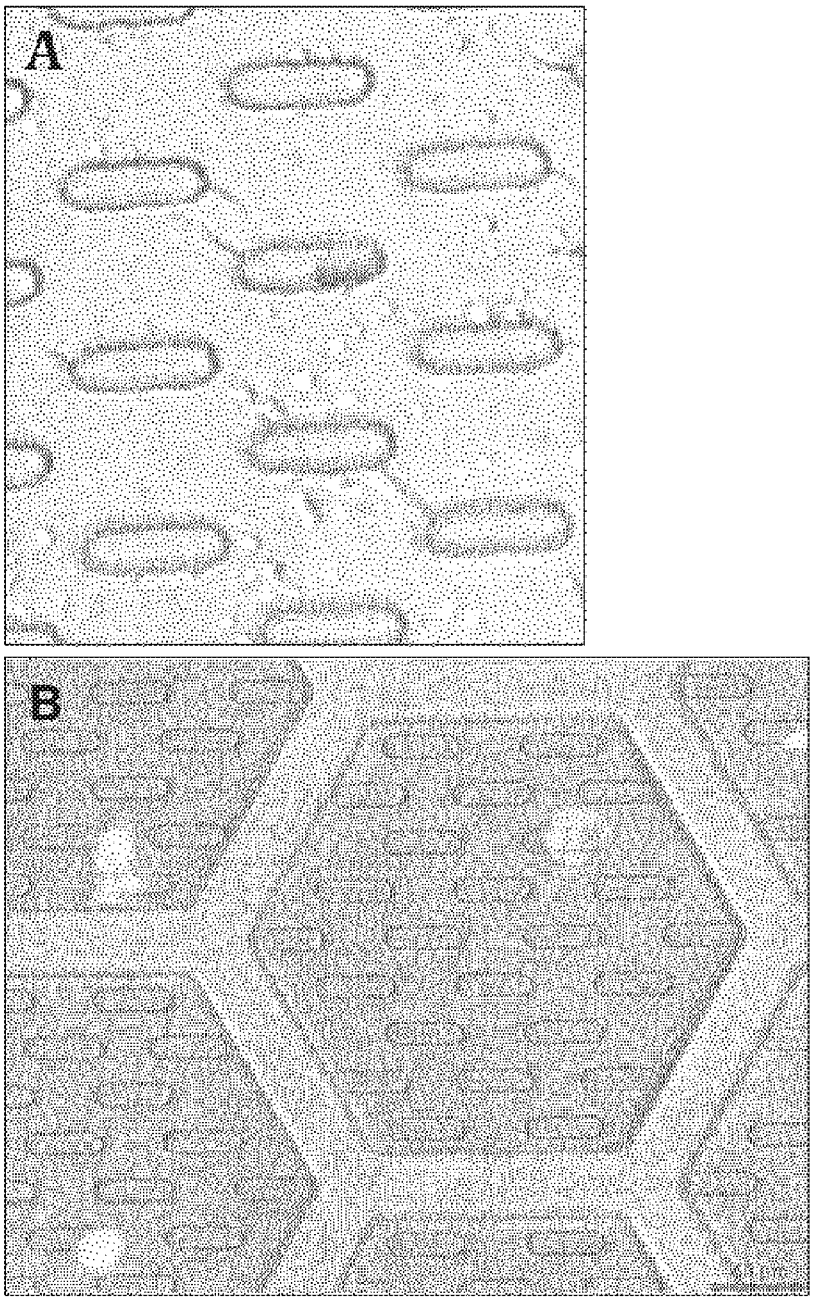

Figure 5

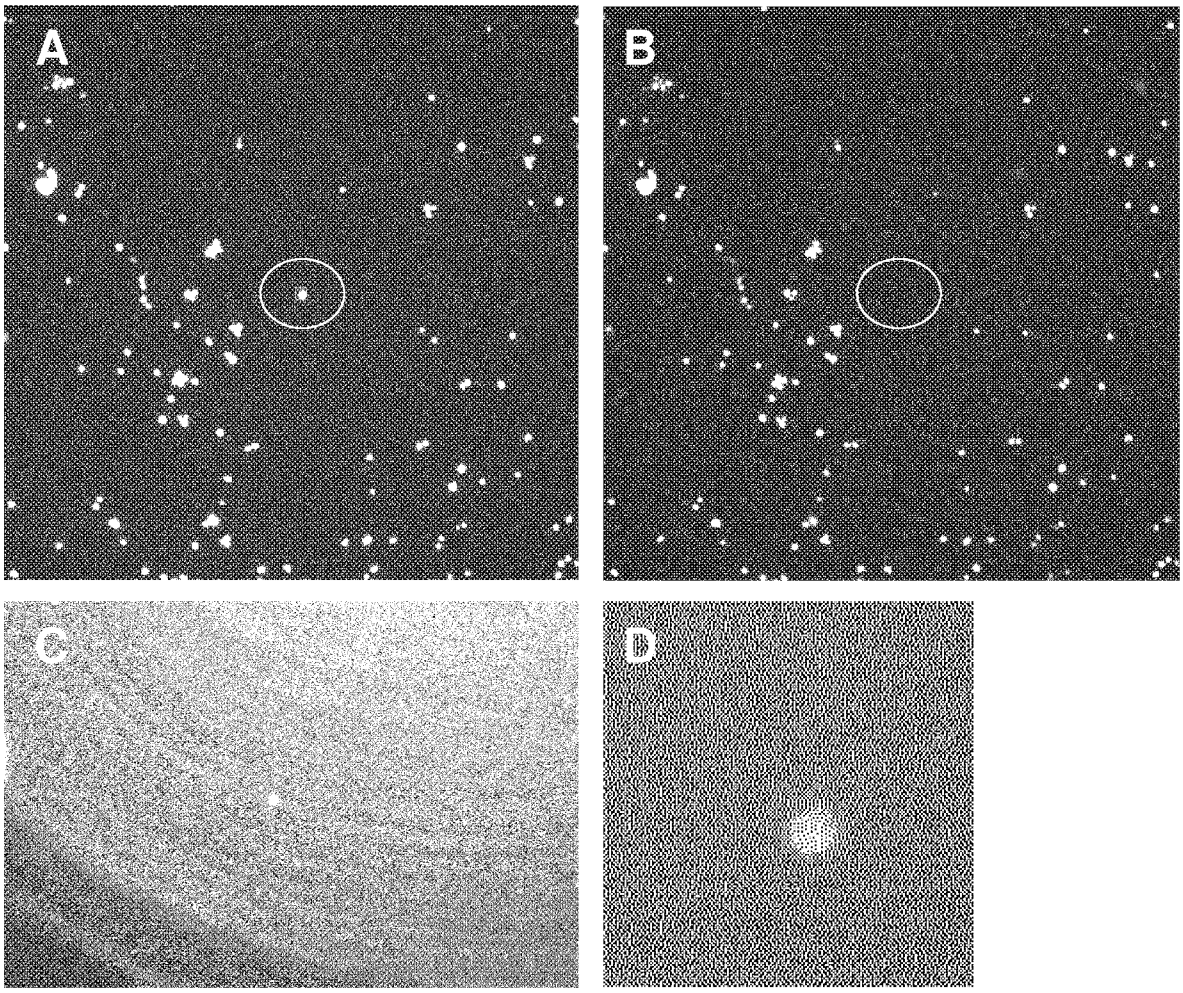

Figure 6

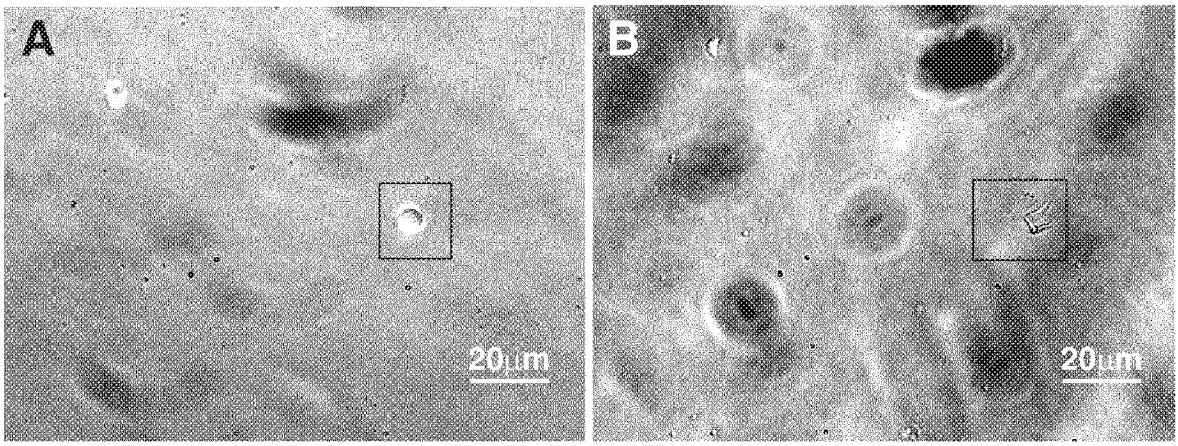

Figure 7

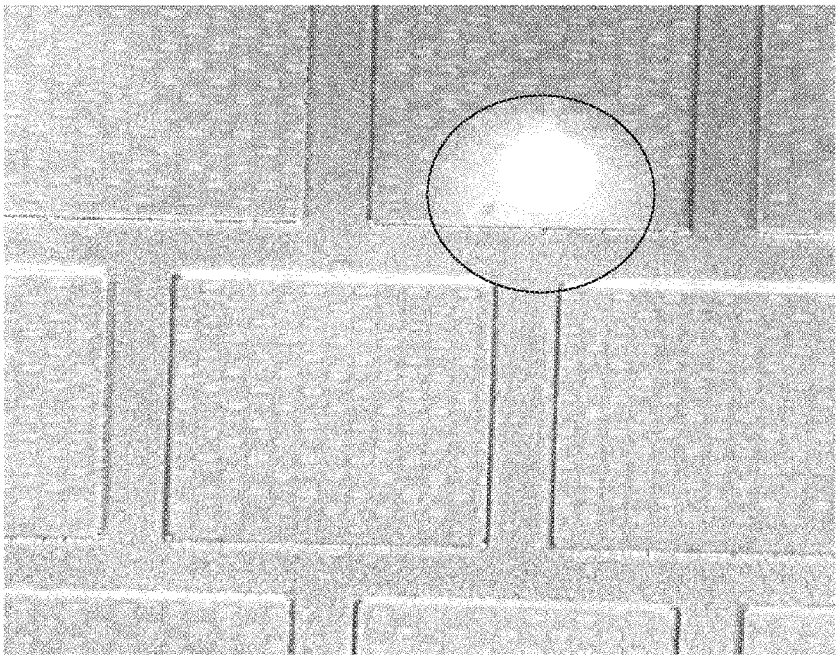

Figure 8

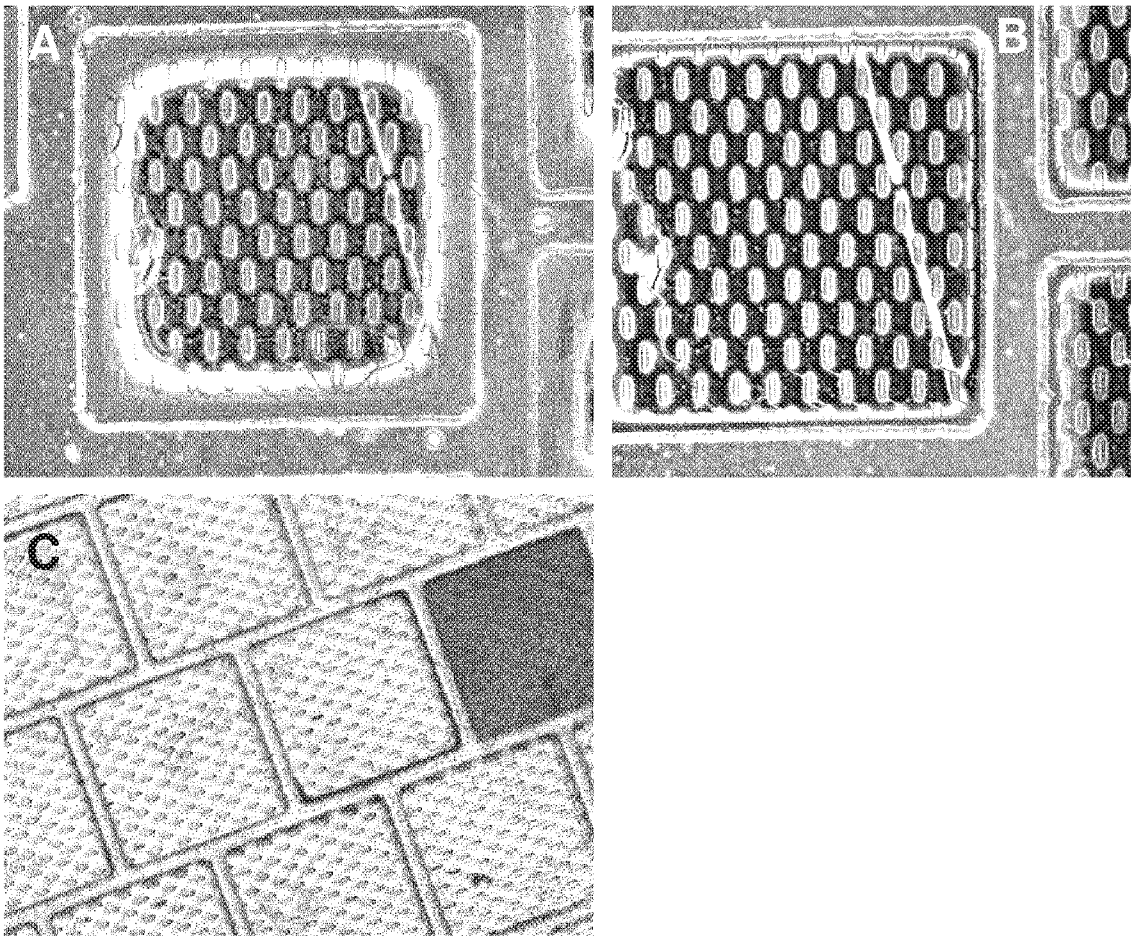

Figure 9

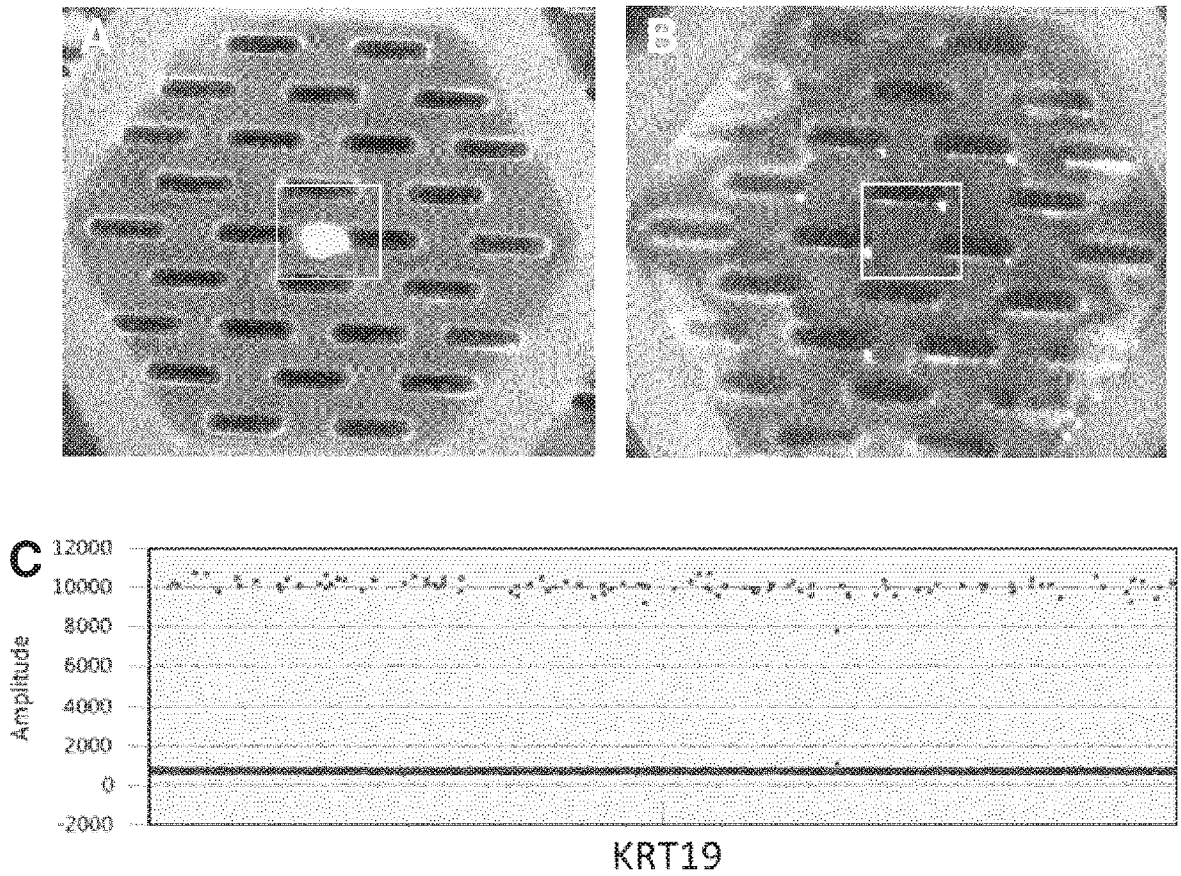

Figure 10

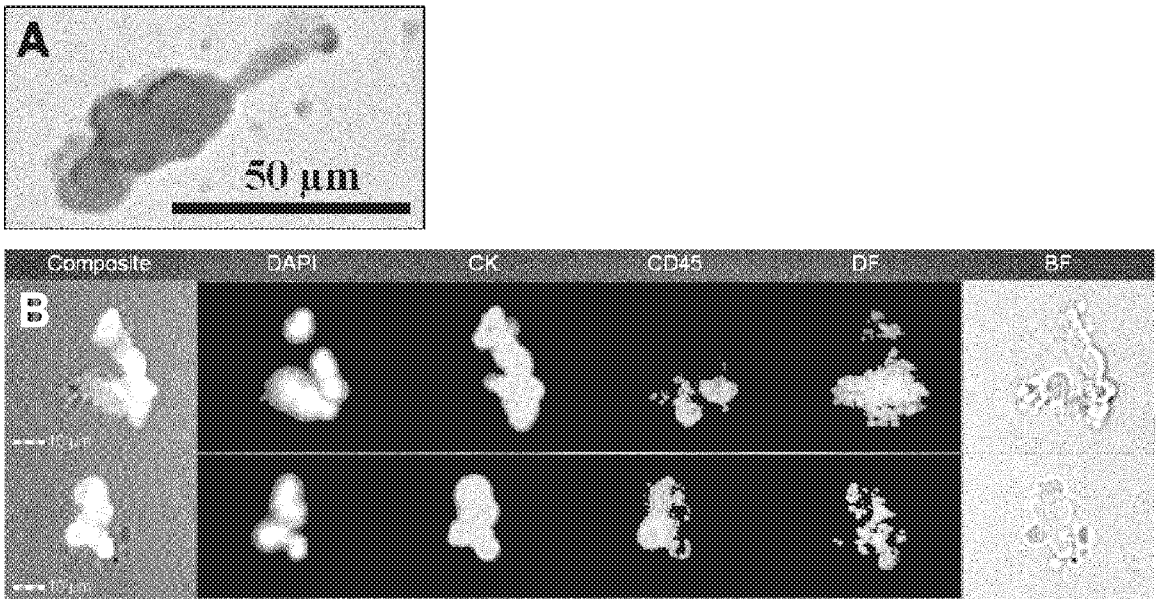

Figure 11

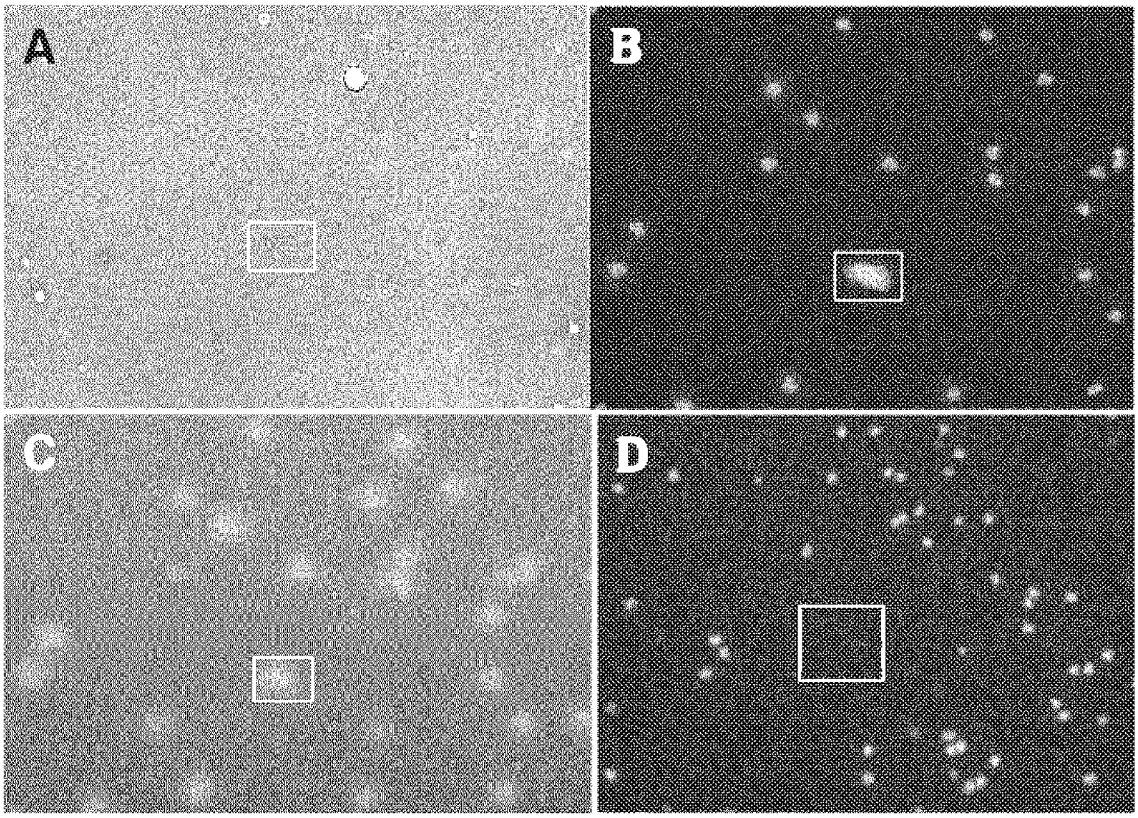

Figure 12

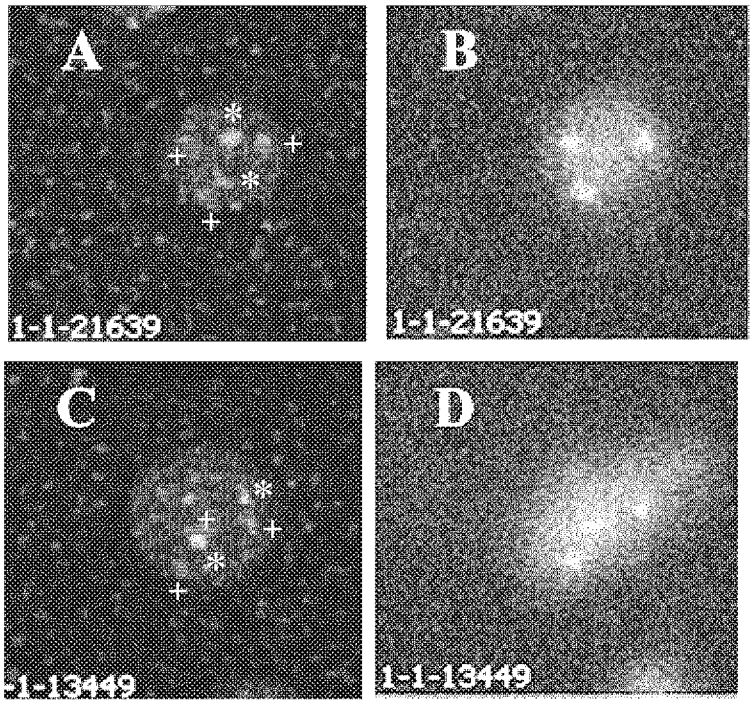

Figure 13

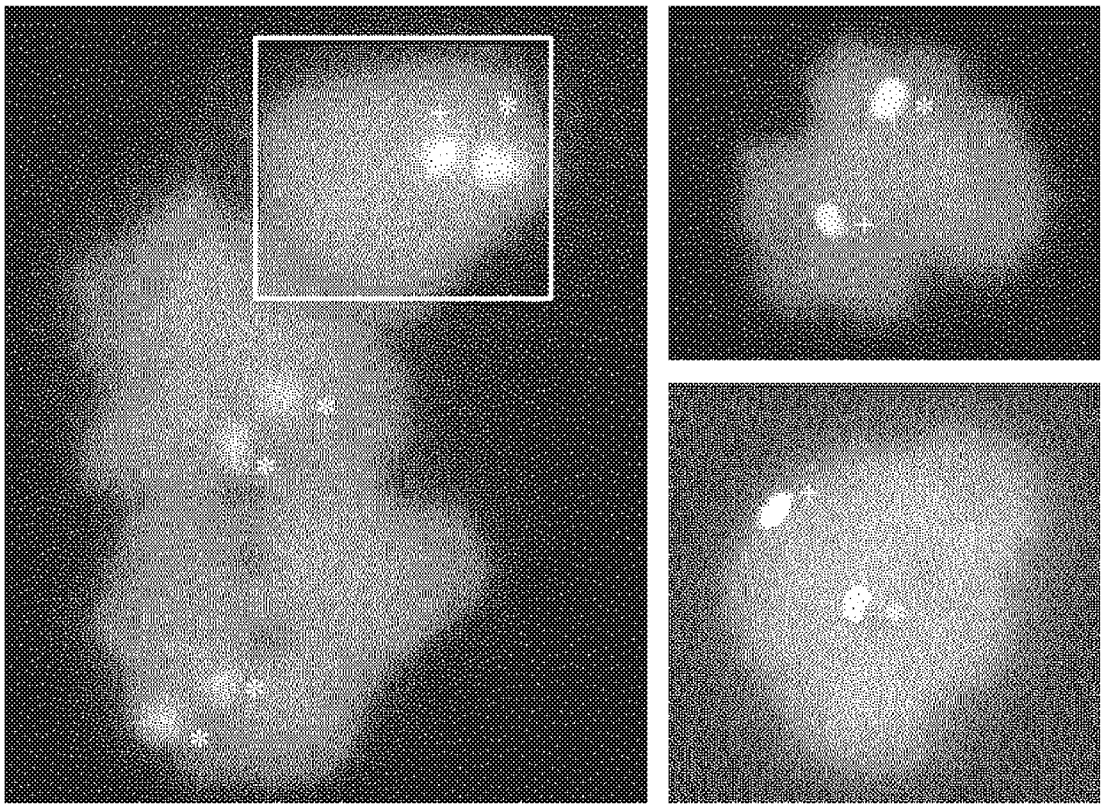

Figure 14

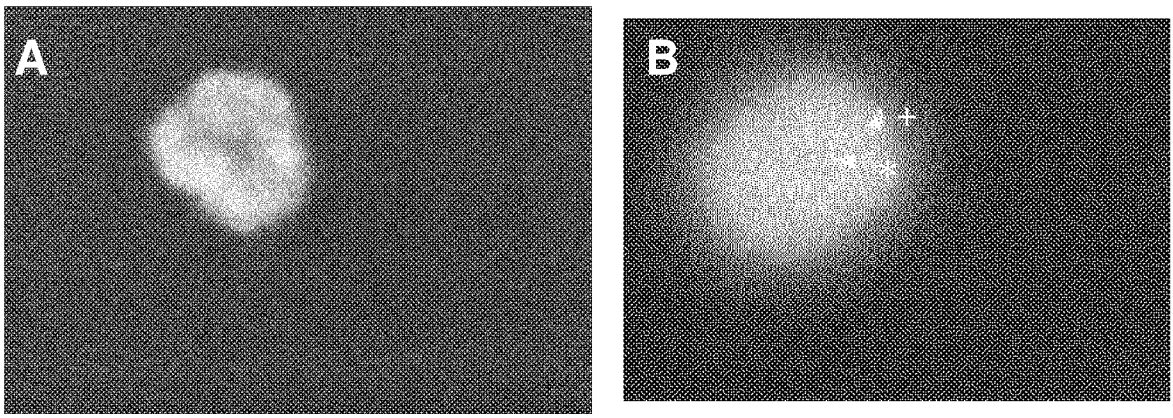

Figure 15

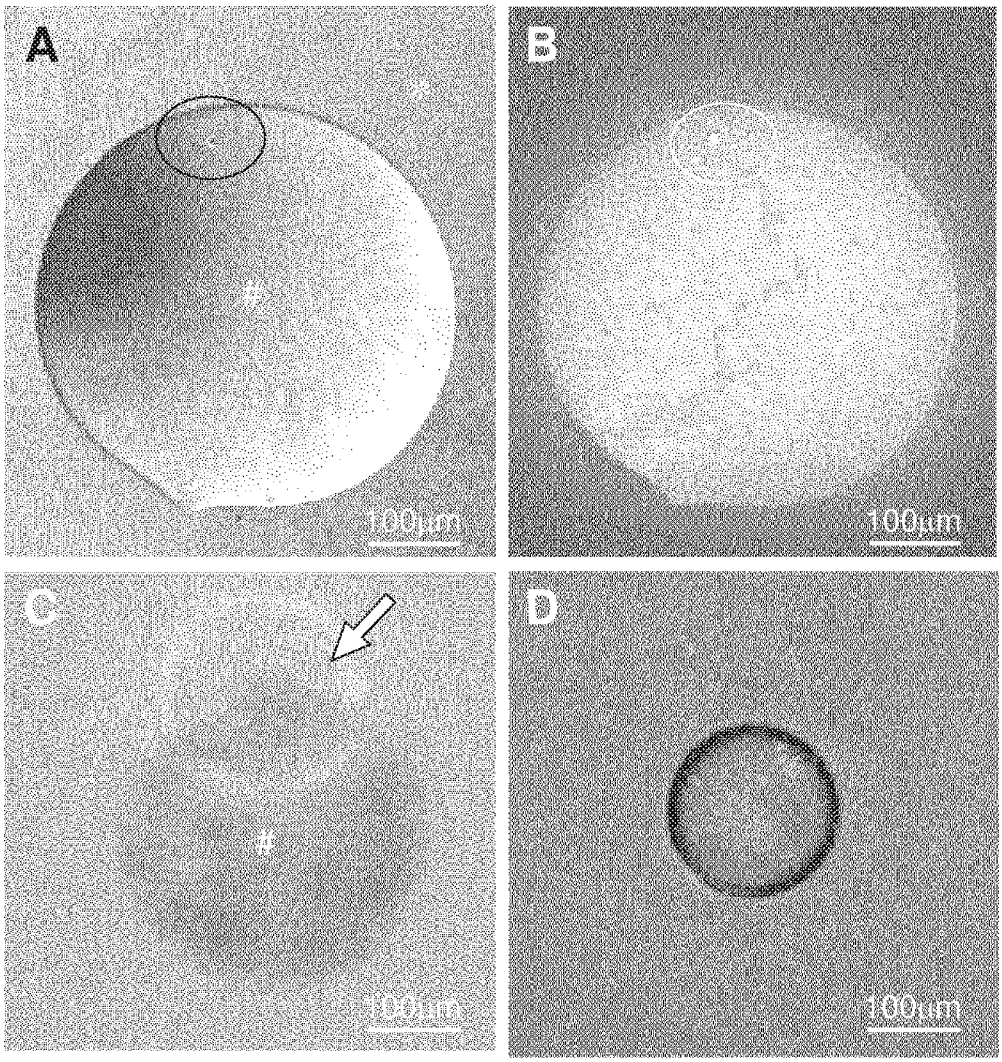

Figure 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2019/000013

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/073 (2010.01) G01N 33/50 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATENW: All english language databases, CAplus, EMBASE, MEDLINE, BIOSIS, INSPEC. Keywords: trophoblasts, inertial microfluidics, DLD, spiral device and like terms.

PATENW:C12N5/073, C12N5/0603, B01L3/5027, B01L2200/027, B01L2200/10, B01L2200/0652, B01L2300/0861, C12N5/0081

Espacenet, Google Scholar, PAMS (NOSE), INTESS: inventor/applicant search.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2019	23 April 2019

Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Lorraine Gunnion AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832575

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2019/000013**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015057159 A1 (CLEARBRIDGE BIOMEDICS PTE LTD) 23 April 2015 Abstract; page 1 line 25; pages 3-4; page 5 lines 5-11; page 6 lines 1-15 & last line; page 7; page 8 lines 29-32; page 18 lines 14-20; page 27 lines 15-31 Figs 3,4 & 7; claims).	1-15
X	WO 2017131580 A1 (CLEARBRIDGE BIOMEDICS PTE LTD) 03 August 2017 Abstract; pages 1-11, claims	1-15
X	WO 2014070776 A1 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 08 May 2014 Abstract; pages 2, 6, 12-13, claims, figures, [0041]	1-15
X	WO 2011109762 A1 (NATIONAL UNIVERSITY OF SINGAPORE et.al.) 09 September 2011 Abstract; pages 2, 8-11, 16-17, 22, 44, 49, 59 Figs 7-9, 14, 16, 17, 20, table 1, claim 23	1-15
X	WO 2008130977 A2 (THE GENERAL HOSPITAL CORPORATION D/B/A MASSACHUSETTS GENERAL HOSPITAL) 30 October 2008 Abstract; pages 3-4; page 9 lines 6-15; paragraph bridging pages 9 & 10; page 22 last line; page 29 lines 23-33; pages 45-46; page 51 lines 11-16; page 52 lines 4-20; examples 5, 14, 16, 18	1-15
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X	KARABACAK, N.M. et al., 'Microfluidic, marker-free isolation of circulating tumor cells from blood samples', Nature Protocols. 2014, vol.9, no.3, pages 694-710 Abstract; page 694 first column first two paragraphs; pages 696-698; Figs 1, 3, 4d	1-2, 4-15
X	US 20100021984 A1 (EDD Jon F et.al.) 28 January 2010 Abstract; [0006], [0008]-[0009], [0025], [0037]-[0046], [0057]	1-15
A	WO 2006108101 A2 (LIVING MICROSYSTEMS et.al.) 12 October 2006 Abstract, pages 2, 6, 10-13,19, 23-24, Example 4, figures 1A-1E, 57; claim 87	1-15
A	WO 2007035498 A2 (LIVING MICROSYSTEMS et.al.) 29 March 2007 Abstract; pages 6-7, 12, 18, 30-40, 45, 47, 55-58, Example 14; Figures 1A-1E, 57A, 71D	1-15
A	WO 2008014516 A2 (LIVING MICROSYSTEMS, INC. et.al.) 31 January 2008 Abstract; [0009], [0011]; [0024]-[0025]; [0068]; [0072]; [0078]; [0083]-[0093]; [0104]; [0116]; Figs 3, 4, 20 A-D, 47-48	1-15
A	VONA, G. et al., 'Enrichment, Immunomorphological, and Genetic Characterization of Fetal Cells Circulating in Maternal Blood', American Journal of Pathology. 2002, vol.160, pages 51-58 page 56	1-15
A	CHOOLANI, M. et al., 'The promise of fetal cells in maternal blood', Best Practice & Research Clinical Obstetrics and Gynaecology. 2012, vol.26, pages 655-667	

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A	HAHN, S. et al. 'Noninvasive prenatal diagnosis of fetal aneuploidies and Mendelian disorders: new innovative strategies', Expert Review of Molecular Diagnostics. 2009, vol.9, pages 613-621	
P,X	WINTER, M. et al. 'Isolation of Circulating Fetal Trophoblasts Using Inertial Microfluidics for Noninvasive Prenatal Testing', Advanced Material Technologies. July 2018, vol.3, no.7, article 1800066 Whole document	1-15
P,X	REZAEI, M. et al. 'A Reappraisal of Circulating Fetal Cell Noninvasive Prenatal Testing', Trends in Biotechnology. Nov 2018, pii: S0167-7799(18)30311-1. doi: 10.1016/j.tibtech.2018.11.001 Figure 3	1-15

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