Supporting Information

Imprinted NanoVelcro Microchips for Isolation and Characterization of Circulating Fetal Trophoblasts – Toward Noninvasive Prenatal Diagnostics

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Preparation of Imprinted PLGA NanoVelcro Substrate

Introduction of PMMA Nanopillar Features onto a Si wafer

Prior to introducing PMMA (polymethyl methacrylate) nanopillar features (200 nm in diameter, 1.5 µm in length, 0.8 µm in spacing) onto Si wafers, the surfaces of the Si wafers were made hydrophilic through the following procedure. The Si wafers were ultrasonicated in acetone and ethanol at room temperature for 10 and 5 min, respectively, to remove contamination from organic grease. The degreased Si substrates were placed in boiling Piranha solution [H_2SO_4/H_2O_2, 4:1 (v/v)] and RCA solution [NH_3/H_2O_2/H_2O, 1:1:5 (v/v/v)] for 1 h each and then rinsed several times with deionized water. The clean Si wafers were spin-coated with 950K PMMA and then patterned and written by electron beam (e-beam) lithography (Leica EBL100 Nanowriter e-beam patterning system with 100 keV beam and laser-controlled stage with resolution down to 30 nm). After developing the pattern at room temperature, the unexposed PMMA was lifted off by immersing the substrate under acetone, leaving PMMA nanopillar feature array on the Si substrate. The procedure of e-beam lithography and Reactive-ion etching (RIE) were illustrated as **Figure S1a-d**.

The Si nanopillar feature array was further triple rinsed with DI water and air dried for 2 min. In order to remove H_2O residue, the Si nanopillar feature array was washed with anhydrous ethanol. Then the Si nanopillar feature array was ready for next step. Silane vapor-phase treatment was carried out on the Si nanopillar feature array with fluoro-silane stock solution (1H, 1H, 2H, 2H-Perfluorooctyltrichlorosilane, 97%, Alfa Aesar Cat # L16606-10G) with a vacuum desiccator at room temperature for 1 h (pumping for 20 min, and then resting under vacuum for 40 min) followed by thermal annealing for 1 h at 70°C. Then the resulting anti-stiction coated Si nanopillar feature master was ready for molding of the PDMS replicates [1].

Fabrication of PDMS replicates

The PDMS base and curing agent was prepared in a 10:1 (w/w) ratio (PDMS pre-polymer) and then well mixed and degased. The resulting pre-PDMS mixture was immediately poured onto the master mold (**Figure S1e**), and a vacuum desiccator was used to help PDMS pre-polymer to fill into the nanostructure of mold master for 1h (pumping and venting by turns for 30 min, and then pumping for 30 min). After being thermal cured inside oven at 70°C for 3 h, the PDMS replicate (**Figure S1f**) was ready for printing NanoVelcro structure on the PLGA.

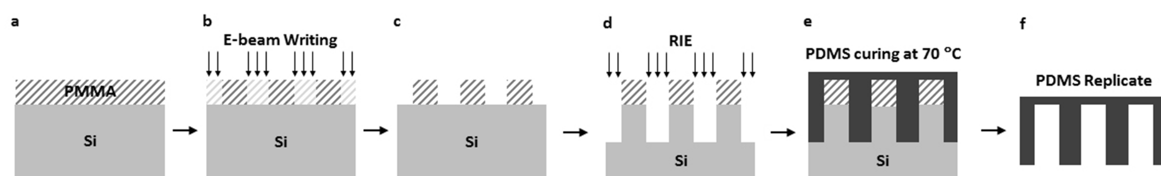

Figure S1. Fabrication of the PMMA nanopillar feature array on a silicon wafer, followed by PDMS Replicate production. **a**, **b**, **c** and **d** PMMA deposition, e-beam lithography, development and RIE etching. **e** and **f** PDMS curing and fabrication of the PDMS replicate.

Fabrication of Imprinted PLGA NanoVelcro substrates

The laser microdissection (LMD) slide (LCM slides: PPS-membrane slides 1.2 μm from Leica) was first oxygen plasma treated for 1 min. PLGA solution (5 wt% in acetonitrile) was then spin-coated (1500 rpm for 30 sec) onto the oxygen plasma treated LCM slide. After being baked on a hot plate at temperature of 120°C for 10-20 sec, the LMD slide coated with PLGA film was ready for nano-imprinting.

The PDMS replicate was first spin-coated with chlorobenzene (1500 rpm for 30 sec.) and then stuck onto the PLGA coated LMD slide. Afterwards, a metal sandwich holder was used to apply a pressure (150 ± 20 g/cm^2) onto the assembled layers, while the imprinting process was carried out at 130°C, a temperature above the glass transition temperature of PLGA. Once the assembled layer cooled down to 20°C, the PDMS replicates were peeled off to give a PLGA NanoVelcro substrate on a LMD slide. Then the imprinted PLGA NanoVelco substrate was ready for the subsequent chemical conjugation [2, 3] (NHS chemistry) to covalently attach streptavidin on the surface.

Optimization and Validation of NanoVelcro Microchips for cTB capture

To optimize and validate the cTB capture performance of the imprinted PLGA NanoVelcro Microchips, we prepared artificial blood samples by spiking JEG-3 cell line cells, pre-stained with DIO (observed under FITC channel), into freshly isolated WBCs derived from healthy donors' blood. Different number of JEG-3 cells of 5, 10, 50, 100, 200 and 500 were separately spiked into individual 5M WBCs (collected from healthy donors' whole blood) suspension solutions in 0.5-mL RPMI. The resulting artificial blood samples were used for following optimization, validation and calibration studies.

cTB Enrichment and Staining

cTB enrichment by gradient centrifuge

Maternal peripheral venous blood samples were obtained and collected in heparin vacutainer tube (10 mL) from the expectant mothers. Before gradient centrifuge, the blood samples were first diluted by ration of 1: 1 with PBS. The gradient separation solution was prepared by adding 2 mL separation solution (Histopaque-1119) in 15 mL centrifuge tubes. Then 6 mL diluted blood sample was carefully placed on the top of the above described gradient separation solutions. After being tightly capped, the tube was then centrifuged at 800 g for 25 min at room temperature. The peripheral blood mononuclear cell (PBMC) layer containing cTBs should be located above Histopaque-1119 separation solution layer. After aspirating and discarding the top layer (plasma), the PBMC layer containing cTB (approximate 2 mL) could be easily collected and transferred to another 15 mL centrifuge tube. Then 9 mL of PBS was added and mixed with the collected cells suspension. The resulting cell suspension was then centrifuged at 300g for 10 min at room temperature. After careful removal of the supernatant, the cell pellet containing cTBs was finally isolated and collected from whole blood for following cTBs capture using the imprinted PLGA NanoVelcro Microchips.

cTB capture and ICC protocol

The artificial blood samples were centrifuged. After carefully discarded the supernatant, the cell pellet were suspended in 200 μL RPMI with 5% FBS. The samples were then introduced into the imprinted NanoVelcro Microchip with anti-EpCAM coated, at the flow rate of 1.0 mL h^{-1}. The capture performance of cTBs was conferred by the interactions between biotin (on the surfaces of cTBs) and streptavidin (on PLGA NanoVelcro Substrates). After on-chip cTB enrichment, a 4-color ICC protocol for parallel staining of Hoechst, anti-CK7 (PE), anti-HLA-G (FITC), and anti-CD45 (TRITC) was employed to identify spiked cTBs and determine the capture efficiencies. The presented technology gave the cTB-capture efficiency of more than 90% reproducibly. In the following on-chip cTB enrichment of real maternal blood samples, the optimized condition of capture agent was used throughout the experiment.

For the maternal blood samples, the gradient centrifuge was carried out on the blood as described in the above section. Then the blood samples with cell pellet containing cTBs was first

re-suspended into 200 μL with RPMI with 5% FBS and then injected into the imprinted PLGA NanoVelcro Microchip at a flow rate of 0.5 mL h^{-1}. After the suspension sample solution containing cTBs fully flew through the imprinted PLGA NanoVelcro Microchip, the on-chip enriched cTBs were first fixed with ethanol (95%) at flow rate of 0.5 mL h^{-1} for 10 minutes. The imprinted PLGA NanoVelcro microchip was briefly washed with washing buffer for 10 min at flow rate of 0.5 mL h^{-1}. Then the captured cTBs were identified by a 4-color ICC protocol for parallel staining of Hoechst, anti-CK7 (PE), anti-HLA-G (FITC), and anti-CD45 (TRITC) to distinguish cTBs from nonspecifically captured WBCs on the NanoVelcro substrates.

After the brief wash with washing buffer, the PDMS chip cover was disassembled from the imprinted PLGA NanoVelcro substrate with immobilized cTBs on the surface. The imprinted PLGA NanoVelcro substrate was then dried in vacuum chamber for 10 min and quantitative image cytometry was conducted by fluorescent microscope (Nikon 90i) to identify cTBs (Hoechst+/CK7+/HLA-G+/CD45-) from nonspecifically captured WBCs (Hoechst+/CK7-/HLA-G-/CD45+).

The complete workflow is summarized in Figure S2.

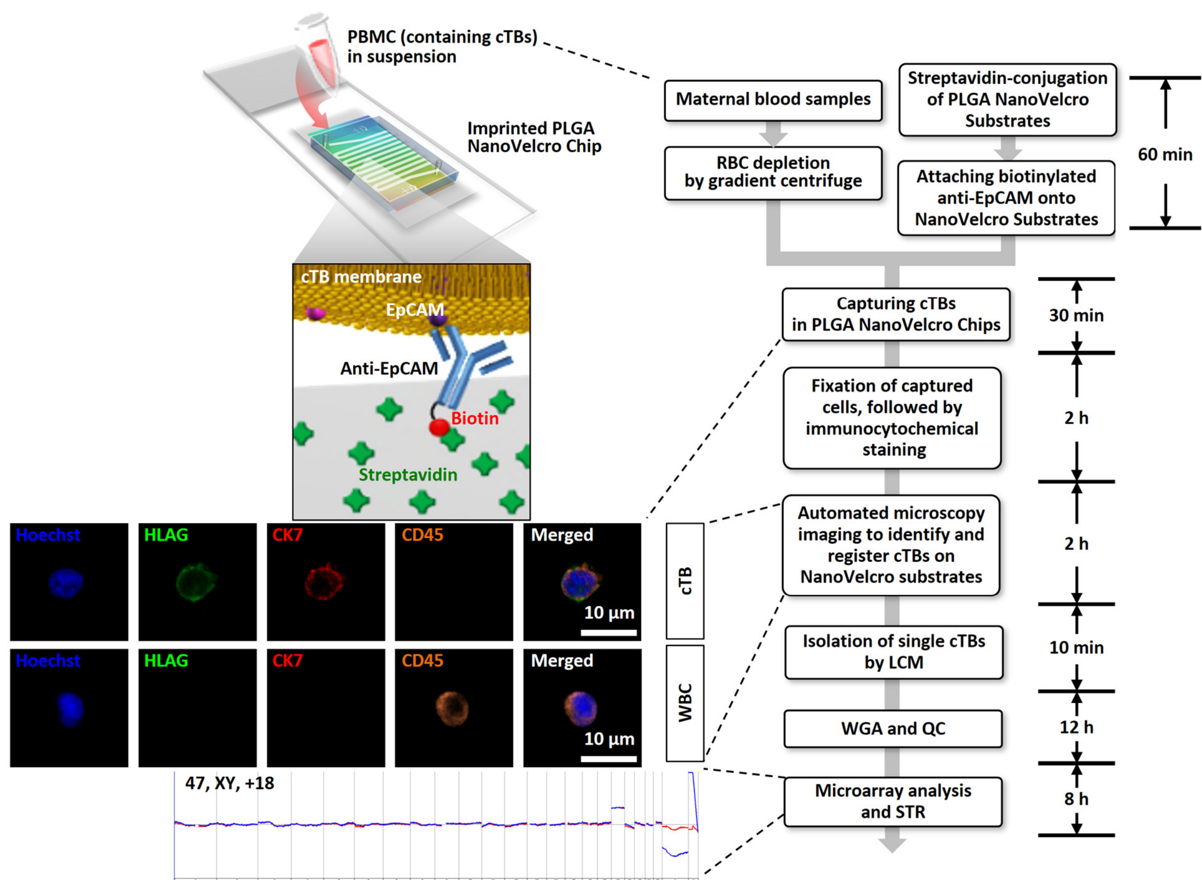

Figure S2. Complete workflow starting from cTB enrichment, via staining, isolation to downstream analysis.

Isolation of Single cTB by Laser Capture Microdissection (LCM)

Before the Laser Capture Microdissection (LCM) of single cTB, the target cTBs (Hoechst+/CK7+/HLA-G+/CD45-) captured by the imprinted PLGA NanoVelcro microchip were first identified and registered using the first fluorescent microscope (Nikon Ni) in conjunction with an auto-scan imaging software (Nikon, Element). Then a second microscope, the ArcturusXT^{TM} LCM System (Applied Biosystems^{TM}) was utilized to selectively dissect identified cTBs. Afterwards, a CapSure^{TM} HS Cap was placed on top of the region of identified cTBs. Then, an 810 nm IR laser beam was applied to melt the polymer membrane on the cap. The resultant conical polymer pillar, so called sticky finger, dropped down and adhered onto the imprinted PLGA NanoVelcro substrate. In the following, a 355 nm UV laser beam was utilized to cut through the imprinted PLGA NanoVelcro substrate in a designed route around cTB

excluding surrounding WBCs. Finally, the dissected cTB was removed from the HS Caps and kept in 4-μL PBSsc (REPLI-g Single Cell Whole Genome Amplification Kit, QIAGEN) in a 0.5 mL PCR tube at -20°C until next step whole genome amplification (WGA, see section 5.2) is performed.

Genetic Characterization of cTBs

Fluorescent in situ hybridization (FISH)

Prior to hybridization, the target cTBs isolated by LCM were placed on a glass slide and incubated for 15 min at 37°C in a pretreatment solution (0.01 M HCl containing 50 μL pepsin) and washed twice in purified water and twice in 1×PBS, followed by 10 min fixation in methanol: acetic acid (3: 1) at 4°C. After two washing steps with 1×PBS and two with purified water, the slides were dehydrated in an ethanol series (70–90–100–100%).

The DNA probes (Vysis, Inc., Downers Grove, IL, USA) used for this study were centromeric DNA probes (chromosome enumeration probe, CEP) for chromosomes X (Green), Y (Red) and 18 (Aqua). The probe mixture consisted of 7 μL CEP buffer, 1 μL CEP X-Spectrum Green, 1 μL CEP Y-Spectrum Red and 1 μL CEP 18-Spectrum Aqua. The FISH procedure was performed according to the protocol recommended by Vysis for directly-labeled probes: the probe mixture was applied to the specimen target area, covered with a glass coverslip and denatured at 75°C on a hot plate for 3 min. The coverslips were sealed with parafilm and hybridization took place overnight in a humidified chamber at 37°C.

For the post-hybridization washing, coverslips were removed and the slides were immersed immediately in 0.4×SSC solution at 73 ± 1°C for 2 min and then washed at room temperature for 1 min with 2×SSC/0.1% NP40 (Tergitol NP40; Sigma) solution. Finally, the slides were mounted in Vectashield antifade medium (Vector Laboratories Inc., Burlingame, CA, USA) containing DAPI counterstain. In order to test the efficiency of the procedure, peripheral blood lymphocytes from a healthy donor (expectant mothers #1, 4-6, see **Table 1)** with a normal (*46, XY*) karyotype was used as a control for each FISH procedure on captured cTBs from maternal blood. The FISH images were taken and processed by Olympus BX61 BioView System (**Figure S3**).

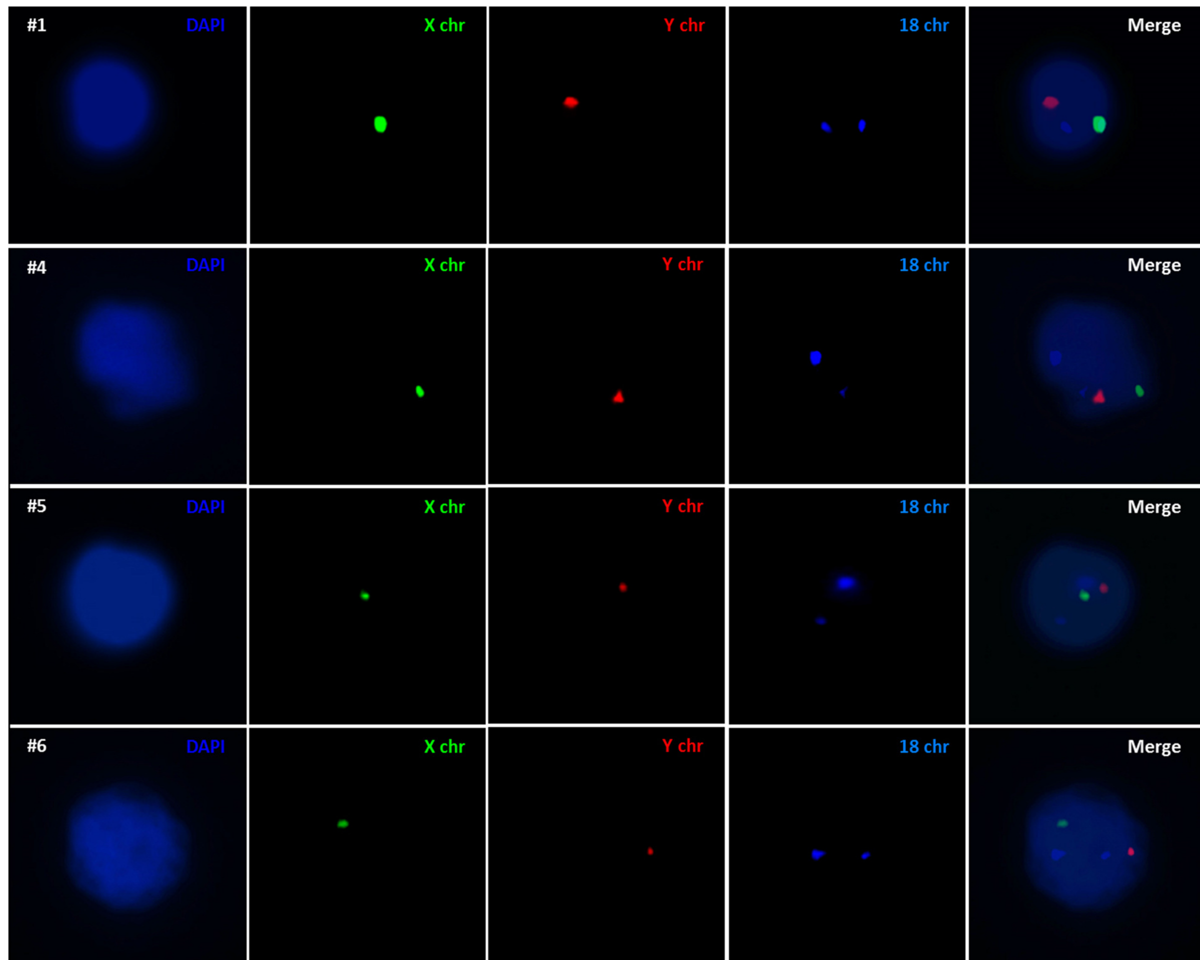

Figure S3. FISH micrographs of an cTB from the respective four expectant mothers (expectant mothers #1, 4-6, **Table 1)** signify one green signal (X chromosome), one red signal (Y chromosome), and two blue signals (chromosome 18).

Whole genome amplification (WGA) of cTBs, followed by microarray analysis

The single cTBs isolated by Laser Capture Microdissection (LCM) was then subjected to subsequent gDNA extraction and amplification using the REPLI-g Single Cell Whole Genome Amplification Kit (QIAGEN, Valencia, CA) according to the recommended standard protocol. After further purification (QIAquick PCR Purification Kit, QIAGEN, Valencia, CA), ca. 1.0 µg of WGA DNA product was applied for microarray analysis (Agilent, SurePrint G3 Human Catalog 8x60K CGH microarrays) [4, 5]. Data were generated and analyzed by PacGenomics (**Figure S4**).

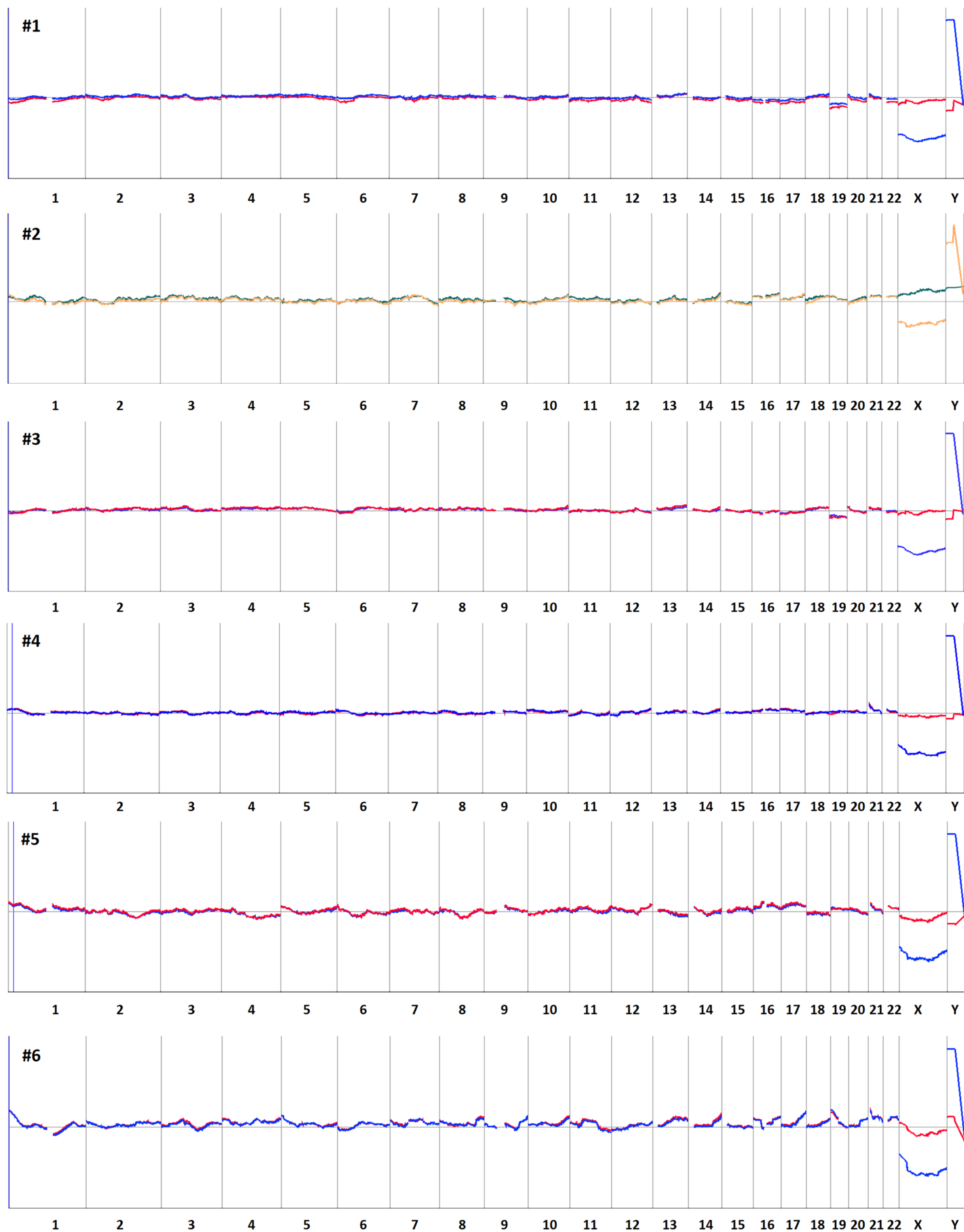

Figure S4. Microarray data obtained for single cTBs from the male fetus carried by each expectant mother (expectant mothers #1-6, see **Table 1**)

Short tandem repeat (STR) genomic fingerprinting

Finally, we utilized short tandem repeat (STR) genomic fingerprinting to establish fetal-maternal relationship between the individually isolated cTB and the matching maternal WBCs. Amplification of STR polymorphisms in both cTBs gDNA samples and maternal gDNA samples (from WBCs) were performed by direct PCR method, which was conducted by using GenePrint® 10 System (Promega, Madison, WI, USA) according to the standard protocol. The GenePrint® 10 System allows co-amplification and three-color detection of ten human loci, including TH01, TPOX, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818, D21S11 as well as Amelogenin. For STR genotyping, the PCR products were subjected to allele analysis at each locus in Genoseq (UCLA Genotyping and Sequencing Core) and identified using Peak Scanner™ Software 2.0 in UCLA Clinical Microarray Core. All samples were tested in duplicate.

References:

1. Qin, D.; Xia, Y.; Whitesides, G. M., Soft Lithography for Micro- and Nanoscale Patterning. *Nat Protoc* **2010**, 5, 491-502.
2. Zhao, L.; Lu, Y. T.; Li, F.; Wu, K.; Hou, S.; Yu, J.; Shen, Q.; Wu, D.; Song, M.; OuYang, W. H.; Luo, Z.; Lee, T.; Fang, X.; Shao, C.; Xu, X.; Garcia, M. A.; Chung, L. W.; Rettig, M.; Tseng, H. R.; Posadas, E. M., High-Purity Prostate Circulating Tumor Cell Isolation by a Polymer Nanofiber-Embedded Microchip for Whole Exome Sequencing. *Adv Mater* **2013**, 25, 2897-2902.
3. Hou, S.; Zhao, L.; Shen, Q.; Yu, J.; Ng, C.; Kong, X.; Wu, D.; Song, M.; Shi, X.; Xu, X.; OuYang, W. H.; He, R.; Zhao, X. Z.; Lee, T.; Brunicardi, F. C.; Garcia, M. A.; Ribas, A.; Lo, R. S.; Tseng, H. R., Polymer Nanofiber-Embedded Microchips for Detection, Isolation, and Molecular Analysis of Single Circulating Melanoma Cells. *Angew Chem Int Ed Engl* **2013**, 52, 3379-3383.
4. Wapner, R. J.; Martin, C. L.; Levy, B.; Ballif, B. C.; Eng, C. M.; Zachary, J. M.; Savage, M.; Platt, L. D.; Saltzman, D.; Grobman, W. A.; Klugman, S.; Scholl, T.; Simpson, J. L.; McCall, K.; Aggarwal, V. S.; Bunke, B.; Nahum, O.; Patel, A.; Lamb, A. N.; Thom, E. A.; Beaudet, A. L.; Ledbetter, D. H.; Shaffer, L. G.; Jackson, L., Chromosomal Microarray versus Karyotyping for Prenatal Diagnosis. *N Engl J Med* **2012**, 367, 2175-2184.
5. Baldwin, E. L.; Lee, J. Y.; Blake, D. M.; Bunke, B. P.; Alexander, C. R.; Kogan, A. L.; Ledbetter, D. H.; Martin, C. L., Enhanced Detection of Clinically Relevant Genomic Imbalances Using a Targeted Plus Whole Genome Oligonucleotide Microarray. *Genet Med* **2008**, 10, 415-429.