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TECHNICAL ADVANCE

Direct Assessment of Single-Cell DNA Using Crudely Purified Live Cells: A Proof of Concept for Noninvasive Prenatal Definitive Diagnosis

Taisuke Sato,*† Yuki Ito,*† Osamu Samura,* Hiroaki Aoki,* Toru Uchiyama,‡ Aikou Okamoto,* and Kenichiro Hata†

From the Department of Obstetrics and Gynecology, The Jikei University School of Medicine, Tokyo; and the Departments of Maternal-Fetal Biology† and Human Genetics,‡ National Research Institute for Child Health and Development, Tokyo, Japan*

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Address correspondence to Kenichiro Hata, M.D., Ph.D., Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan. E-mail: hata-k@ncchd.go.jp.

Noninvasive testing techniques are often used for fetal diagnosis of genetic abnormalities but are limited by certain characteristics, including noninformative results. Thus, novel methods of noninvasive definitive diagnosis of fetal genetic abnormalities are needed. The aim of this study was to develop a single-cell DNA analysis method with high sensitivity and specificity that enables direct extraction of genetic information from live fetal cells in a crude mixture for simultaneous evaluation. Genomic DNA from circulating fetal $CD45^{-}CD14^{-}$ cells, an extremely rare cell type, extracted from 10-mL samples of maternal peripheral blood, was extracted using a single-cell–based droplet digital (sc-dd) PCR system with a modified amount of polymerase. A hexachloro-6-carboxyfluorescein–labeled *RPP30* probe was used as an internal control and a 6-carboxyfluorescein–labeled *SRY* probe as a target. The results indicated that no droplets generated with samples from pregnant women carrying female fetuses were positive for both probe signals, whereas droplets prepared with samples from pregnant women carrying male fetuses were positive for both probe signals. The latter was considered a direct assessment of genetic information from single circulating male fetal cells. Thus, the modified sc-ddPCR system allows the detection of genetic information from rare target cells in a crudely purified cell population. This research also serves as a proof of concept for noninvasive prenatal definitive diagnosis. *(J Mol Diagn 2020, 22: 132–140; https://doi.org/10.1016/j.jmoldx.2019.10.006)*

Prenatal genetic testing using circulating cell-free DNA is one of the most successful genetic screening tools. Since circulating cell-free DNA, which reflects genetic information of placental villi, has been proved to exist in maternal peripheral blood,[1–3] methods of assessing fetal genetic information noninvasively have become increasingly widespread in laboratory medicine. Currently, the noninvasive prenatal test is clinically applied for assessing fetal aneuploidies with high sensitivity and specificity.[4] However, cases in which the noninvasive prenatal test results are discordant with the fetal genetic information have been reported.[5–7]

The presence of fetal cells in maternal blood circulation was reported before the discovery of circulating cell-free DNA in maternal peripheral blood.[8,9] Therefore, many researchers have investigated the purification of these circulating fetal cells using various methods.[8–17] However, circulating fetal cells among maternal nucleated blood cells are extremely rare, two to six fetal cells in 1 mL of maternal blood.[18] Therefore, most methods require cell fixation or cell staining to purify a high concentration of fetal cells. Moreover, a multicenter phase II trial concluded that it was difficult to clinically apply circulating fetal cells for detecting fetal genomic information because of the high false-positive rates in confirming circulating fetal cells which were isolated by fluorescence-activated cell sorting and magnetic-activated cell sorting (MACS) from maternal blood samples.[19]

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We previously reported on a single-cell–based droplet digital (sc-dd) PCR system that analyzes single-cell DNA without whole-genome amplification, cell fixation, or cell staining.[20] With this sc-ddPCR system, numerous cells are simultaneously separated and analyzed by encapsulation in a droplet that contains digital PCR reagents. In this previous study, we successfully analyzed single-cell genomic DNA from cultured cells and nucleated blood cells that were highly purified using fluorescence-activated cell sorting.[20] However, sc-ddPCR has not been applied to cruder cell suspensions because the impurities therein decrease the reaction efficiency of multiplex PCR or increase the fragility of each droplet.

In the current study, this sc-ddPCR system was modified to enable the assessment of single-cell–level genomic DNA with higher sensitivity. To clearly confirm the sensitivity of this modified sc-ddPCR system, the genomic DNA of circulating male fetal cells was detected in a crudely sorted cell suspension derived from peripheral blood samples from mothers with male fetuses at the single-cell level. This study serves as a proof of concept for noninvasive prenatal diagnosis using circulating fetal cells without any strict cell purification.

Materials and Methods

Ethics Statement

This research was approved by the institutional review boards of the National Center Institute for Child Health and Development (Tokyo, Japan; IRB number 699) and the Jikei University School of Medicine [IRB number 27-059 (7944); Tokyo, Japan]. Detailed cytogenetic and molecular genetic analyses were performed with written informed consent from both parents.

Sampling and Extraction of Nucleated Blood Cells

A total of 32 pregnant women without any obstetrical complications or abnormalities and malformations as determined by fetal ultrasonography at 20 weeks of gestation were recruited. A 10-mL sample of peripheral blood was obtained from each pregnant woman using blood-collection tubes containing EDTA-2K. These blood samples were diluted threefold using 1× phosphate-buffered saline (PBS). Diluted blood samples were overlaid onto 3 mL of Percoll solution (GE Healthcare, Pittsburgh, PA) with 1.119 g/mL (520 mOsm/L) density and centrifuged at 400 × *g* for 30 minutes at room temperature to eliminate non-nucleated erythrocytes.[21] The buffy coat on the Percoll solution layer was collected and resuspended with 1× PBS with 0.1% bovine serum albumin after filtering through a 0.35-μm membrane filter. This cell suspension was centrifuged at 400 × *g* for 5 minutes at room temperature. To completely remove non-nucleated red blood cells, the washed cells were resuspended with 4 mL of BD Pharm Lyse lysing buffer (BD Biosciences, San Jose, CA) and incubated for 15 minutes at room temperature. After centrifugation at 400 × *g* for 5 minutes, the nucleated blood cells were washed twice with autoMACS Running Buffer (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) and finally resuspended with 80 μL of autoMACS Running Buffer.

MACS

The washed nucleated blood cells were mixed with 10 μL each of CD45 microbeads and CD14 microbeads (Miltenyi Biotech) and incubated for 15 minutes at 4°C. After adding 900 μL of autoMACS Running Buffer, the cell suspension was centrifuged at 300 × *g* for 10 minutes at 4°C to remove the extra microbeads. The cells were resuspended in 500 μL of autoMACS Running Buffer and dropped into the LD column (Miltenyi Biotech) in the magnetic field to manually perform MACS. Finally, the $CD45^{-}CD14^{-}$ cell fraction was collected, white blood cells and large cells were removed to align the cell size, and cells were washed twice using 1× PBS.

Confirmation of the Cell Population after MACS

Cells sorted by MACS were resuspended in 100 μL of 1× PBS and adhered to Shiran-coated slides (Muto Pure Chemicals, Tokyo, Japan) by centrifugation for 5 minutes at 800 rpm using the Cytospin 3 cytocentrifuge (Thermo Fisher Scientific/Shandon Scientific). After rapid air-drying, cells were stained with May-Grünwald Giemsa reagent (Muto Pure Chemicals) for 5 minutes at room temperature. After washing with 1/150 mol/L phosphate buffer (pH = 6.4) for 2 minutes, cells were stained with 1:20 diluted Giemsa reagent (Muto Pure Chemicals) for 30 minutes at room temperature. After another wash in running tap water and rapid air-drying, the cells were covered with a Micro Cover Glass (18 mm × 18 mm; Matsunami Glass USA, Bellingham, WA) using a drop of Mount Quick mounting solution (Daido Sangyo, Tokyo, Japan). The stained cells were observed using the Axio Imager 2 fluorescence microscope system (Carl Zeiss Microscopy, Jena, Germany).

sc-ddPCR

sc-ddPCR was performed according to a previously described method (Figure 1).[20] For a reference signal, a hexachloro-6-carboxyfluorescein–labeled *RPP30* probe and primer set was used, which was the same as that used in the original method,[20] and for target, 1 μL of TaqMan Copy Number Assay (catalog number Hs01026408_cn; Thermo Fisher Scientific, Waltham, MA) was used, which contained a 6-carboxyfluorescein–labeled *SRY* probe and primer set. One unit per well of the KAPA2G Robust HotStart DNA polymerase (KAPA Biosystems, Wobum, MA) in the modified sc-ddPCR system, whereas we added 4 units per well of the KAPA2G Robust HotStart DNA polymerase in

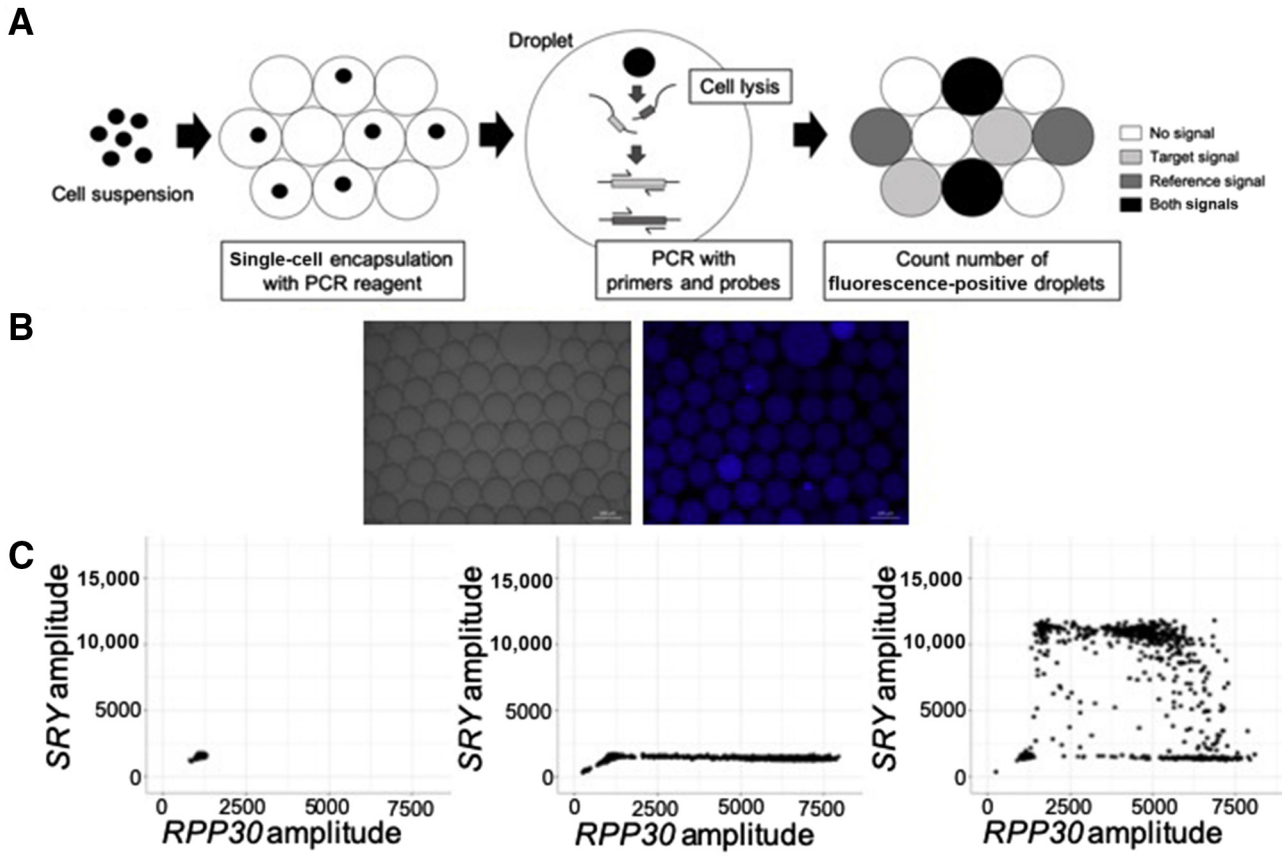

Figure 1 Single-cell–based droplet digital (sc-dd) PCR system. **A:** Schema of our sc-ddPCR system, which was adapted from Igarashi et al (2017).[20] Using the QX200 ddPCR system, each cell is simply encapsulated in one droplet. Up to 3000 cells per well can be individually encapsulated. Subsequently, cell lysis and PCR with probes and primers are performed in each droplet. Last, by quantifying the fluorescent droplets, single-cell genomic DNA can be assessed. **B:** Confirmation of single-cell encapsulation after generating droplets with a human B-cell line[22] using our modified sc-ddPCR system. **Left**, in bright field; **right**, with a Hoechst filter. **C:** Two-dimensional plot of signal amplitude of *SRY* and *RPP30* probes in each droplet detected using our modified sc-ddPCR system. **Left**, signal amplitude of probes with 1× phosphate-buffered saline as a blank control. **Middle**, signal amplitude of probes with a human female B-cell line (HEV0230) as a negative control.[22] **Right**, signal amplitude of probes with a human male B-cell line (HEV0057) as a positive control.[22] Original magnification: ×400 (**B**).

the original sc-ddPCR system.[20] To generate PCR mix containing additional DNA polymerase and the TaqMan primers and probe sufficient for 20 μL reactions in each well, the volume of the cell suspension was adjusted using 1× PBS. For each maternal blood sample, we used 93 wells to analyze all of the cells after performing MACS. In addition, 3 control wells were prepared for the determination of the thresholds of both probe signals: a positive-control well with the human Japanese male B-cell line HEV0057,[22] a negative-control well with the human Japanese female B-cell line HEV0230,[22] and a blank-control well with only 1× PBS. To minimize the effect of detergents on the cells and TaqMan probe in each droplet, we generated droplets for 24 wells at a time using the QX200 Droplet Generator (Bio-Rad, Hercules, CA) just after mixing all of the reagents with the cell suspension.[20] After the generation of droplets, we performed PCR and subsequently analyzed the signal of each droplet using the QX200 Droplet Reader (Bio-Rad). The number of generating droplets were analyzed with the *t*-test in statistical software package R version 3.5.2 (*https://www.r-project.org*). Two-dimensional plots, boxplots, and a bar plot in this study were generated with the R package ggplot2.

Confirmation of Single-Cell Encapsulation in Each Droplet

To confirm single-cell encapsulation in the modified sc-ddPCR system, droplets were generated with human B-cell lines (HEV190 and HEV230)[22] and 10 μg of Hoechst 33528 (Sigma-Aldrich, St. Louis, MO). Immediately thereafter, droplets were observed using the Axio Imager 2 fluorescence microscope system (Carl Zeiss Microscopy).

DNA Extraction from Cell Suspensions or Umbilical Cord Blood Samples

DNA was extracted from each of the cell suspensions or umbilical cord blood samples using the QIAamp DNA Blood Midi Kit (Qiagen, Venlo, Netherlands) or DNA

Table 1 Detailed Results for the Single-Cell–Based Droplet Digital PCR Analyses, Which Were Performed with Different Amounts of DNA Polymerase with a Human Male B-Cell Line (HEV0057)

Enzyme amount	Total inputted male cell count	$SRY^{+}RPP30^{+}$ droplets	$SRY^{+}RPP30^{-}$ droplets	$SRY^{-}RPP30^{+}$ droplets	$SRY^{-}RPP30^{-}$ droplets	Total droplets
Original system (4 units per well)	5682	607	122	328	27,931	28,988
Modified system (1 unit per well)	5682	1330	117	184	37,435	39,066

Blood Mini Kit (Qiagen) following the manufacturer's protocol. The concentration of extracted DNA was measured using the Qubit 2.0 fluorometer (Invitrogen Life Technologies, Carlsbad, CA).

Genetic Confirmation of Circulating Male Fetal Cells in the $CD45^{-}CD14^{-}$ Cell Fraction

Because the presence of circulating fetal cells is extremely rare, we extracted genomic DNA from the $CD45^{-}CD14^{-}$ cell fraction and detected the presence of the *SRY* gene using ddPCR. The genomic DNA, which was diluted to below 10 ng per well with 1× PBS, was mixed with 10 μL of ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 1 μL of TaqMan Copy Number Assay (catalog number Hs01026408_cn; Thermo Fisher Scientific), and the same *RPP30* probe and primer set used in the sc-ddPCR system[20] (250 nmol/L of the probe and 750 nmol/L of each primer) in a 20-μL volume of PCR mix per well. Droplets were generated with 75 μL of Droplet Generation Oil for Probes (Bio-Rad) using the QX200 Droplet Generator (Bio-Rad). PCR was performed using the following thermocycling parameters: 10 minutes at 95°C to activate the enzyme in the ddPCR Supermix for Probes (No dUTP), followed by 39 cycles of 30 seconds at 94°C and 1 minute at 60°C.

PCR of the *SRY* Gene

To confirm the sex of the fetuses, we evaluated the presence of the *SRY* gene in the genomic DNA extracted from each umbilical cord blood sample using real-time PCR. Real-time PCR was performed with 10 μL of Genotyping Master Mix (Thermo Fisher Scientific) and the same probe and primer sets used in sc-ddPCR in a 20-μL volume of PCR mix per well. PCR was performed using the following thermocycling parameters: 10 minutes at 95°C to activate the enzyme in the Genotyping Master Mix, followed by 40 cycles of 15 seconds at 96°C and 1 minute at 60°C.

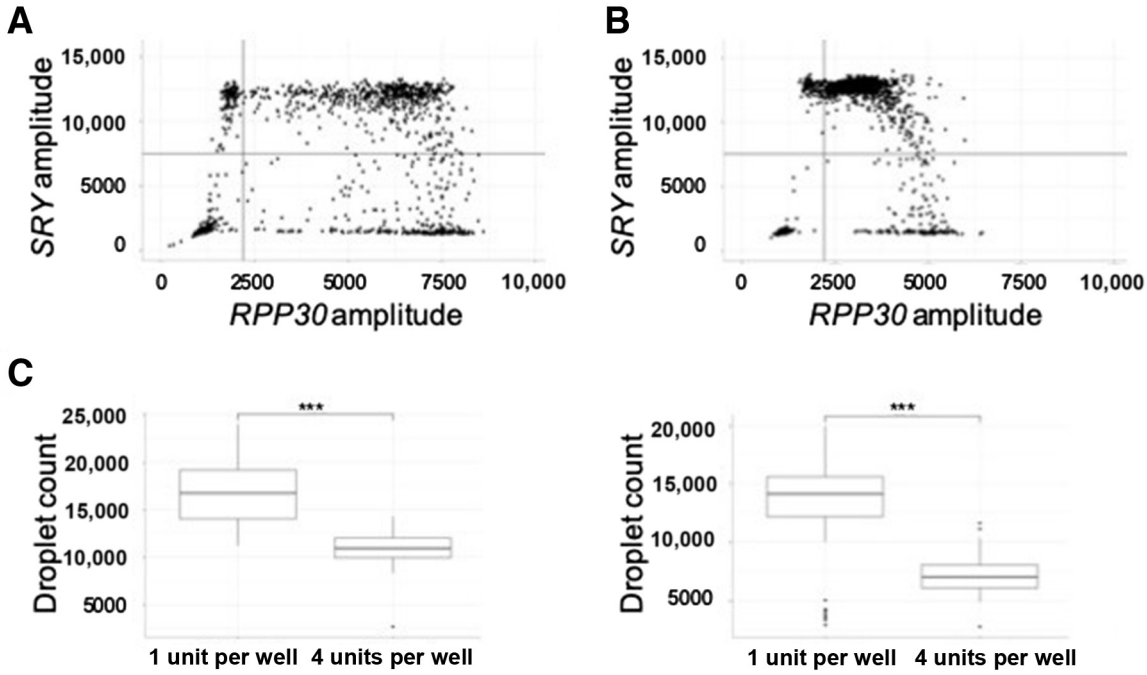

Figure 2 Modification of the single-cell–based droplet digital (sc-dd) PCR system for the detection of rare cells with a higher sensitivity. sc-ddPCR was performed with different amounts of DNA polymerase using a human male B-cell line (HEV0057) in every 3 wells as a control. **A** and **B:** Two-dimensional plots of signal amplitude of *SRY* and *RPP30* probes in each droplet using 4 units per well of KAPA2G Robust HotStart DNA (KAPA Biosystems, Wobum, MA) polymerase according to the original sc-ddPCR system (**A**) and 1 unit per well of KAPA2G Robust HotStart DNA polymerase as in the modified sc-ddPCR system (**B**). These plots show the merged results of 3 wells with each sc-ddPCR system. Detailed results of these sc-ddPCR findings are shown in Table 1. The **bold lines** show thresholds for amplification of *SRY* and *RPP30*, which were determined using control samples. **C:** Boxplots showing the assessment of droplet numbers generated from 1 well according to enzyme amount. **Left**, the number of droplets per well generated with 1× phosphate-buffered saline (1 unit per well, 93 wells; 4 units per well, 67 wells). **Right**, the number of droplets per well generated with the $CD45^{-}CD14^{-}$ cell fraction of maternal peripheral blood samples (1 unit per well, 17 wells; 4 units per well, 30 wells). $***P < 0.001$.

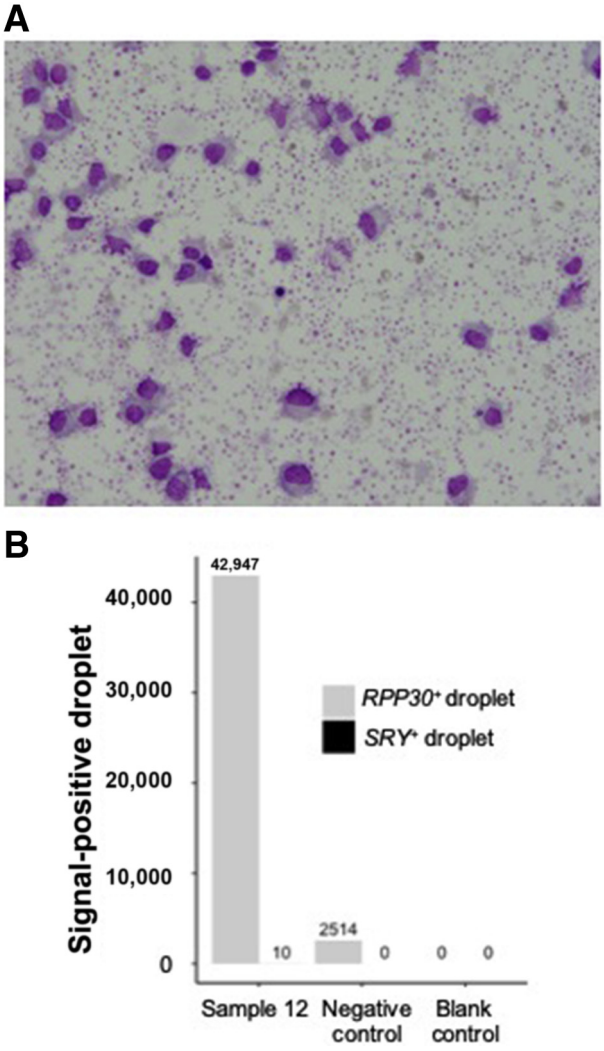

Figure 3 Extraction of $CD45^{-}CD14^{-}$ cells from maternal peripheral blood using the magnetic-activated cell sorting protocol. **A:** The $CD45^{-}CD14^{-}$ cell population in 10 mL of maternal peripheral blood stained with May-Grünwald Giemsa (×400 in bright field). Nucleated red blood cells were mixed with plasmacytes, immature neutrocytes, and highly concentrated platelets. **B:** Copy number analysis using the droplet digital PCR to genetically confirm the presence of fetal cells in the $CD45^{-}CD14^{-}$ cell fraction extracted from peripheral blood from mothers with male fetuses (number 16 in Table 2).

Results

Modification of sc-ddPCR for Higher Sensitivity

To assess the single-cell genomic DNA of extremely rare cells in crude cell suspensions, we required a more sensitive sc-ddPCR system than the original system owing to the contamination in each droplet.[20] We reduced the KAPA2G Robust HotStart DNA polymerase added to sc-ddPCR to 1 unit per well. In the sc-ddPCR examination using the human male B-cell line, the modification reduced the number of droplets with signal corresponding to either *RPP30* or *SRY*, whereas the number of droplets with both signals was increased approximately twofold (Table 1 and Figure 2, A and B). The number of $SRY^{+}RPP30^{+}$ droplets was increased by approximately 10% using the modified sc-ddPCR system compared with the conventional sc-ddPCR system. In addition, the number of generated droplets was increased (Table 1). These results showed that this modification improved the multiplex PCR efficiency in each droplet and that the enzyme reduction contributed to a higher sensitivity of the system. The intensities of both signals tended to be lower with the modified sc-ddPCR system, implying that cruder cell-suspension samples could be analyzed while maintaining the high sensitivity and specificity. This sc-ddPCR system successfully assessed each cell in the $CD45^{-}CD14^{-}$ fraction of the peripheral blood samples from a male adult. Furthermore, similar to the cell-line examination, more analyzable droplets were obtained from the crudely sorted samples using this modified system (Table 1 and Figure 2C).

Crude Purification of Extremely Rare Cells in Circulation

To verify the high sensitivity of our modified sc-ddPCR system under crude conditions, we investigated the single-cell genomic DNA of circulating fetal cells, which have been reported to be not only extremely rare in maternal blood circulation but also fragile.[18,23] Therefore, we collected the $CD45^{-}CD14^{-}$ cell fraction from maternal peripheral blood samples by manual MACS. Approximately 99% of nucleated blood cells were removed with our method, yielding relatively uniformly sized cells. In this fraction, nucleated red blood cells, which are one typical type of fetal cells in maternal blood circulation, along with plasmacytes, immature neutrocytes, and highly concentrated platelets, were detected histologically with May-Grünwald Giemsa staining (Figure 3A). Furthermore, the genomic DNA that was extracted from $CD45^{-}CD14^{-}$ cells in a maternal peripheral blood sample corresponding to a male fetus was genetically confirmed to contain a few copies of the *SRY* gene (Figure 3B and Table 2). We therefore determined that our purification method was effective for the isolation of the cell fraction containing circulating fetal cells. However, the $CD45^{-}CD14^{-}$ fraction, which was extracted from 10 mL of maternal peripheral blood, contained $1.89 \pm 1.20 \times 10^{5}$ cells, which mainly consisted of concentrated platelets (Table 2). To simultaneously analyze numerous crudely separated cells, we modified the sc-ddPCR system to include 93 experimental wells and 3 control wells. As a result, adequate signals of both genes were obtained from each acceptable droplet with the modified sc-ddPCR system.

Detection of Single-Cell Genomic DNA from Extremely Rare Circulating Cells

Finally, we analyzed 13 maternal peripheral blood samples with the modified sc-ddPCR system (Table 3). The total number of acceptable droplets in each sample was

Table 2 Cell Counts at Every Step of Cell Purification from 10-mL Maternal Peripheral Blood Samples Using Magnetic-Activated Cell Sorting.

Sample no.	Maternal age, years	Fetal sex on ultrasonography	Gestational age, weeks	Gravida	Parity	Total PBMCs in 10 mL of peripheral blood ($\times 10^5$ cells)	$CD45^-CD14^-$ cells in 10 mL of peripheral blood ($\times 10^5$ cells)
1	27	Male	21.86	2	1	140	4.00
2	35	Male	26.43	2	1	192	0.28
3	34	Male	33.14	2	0	141	0.66
4	34	Male	26.14	2	1	118	0.89
5	36	Male	34.29	1	0	144	1.09
6	38	Male	27.86	2	1	130	1.12
7	35	Male	33.86	2	1	123	2.56
8	32	Female	34.29	1	0	128	1.11
9	35	Male	33.00	1	0	244	1.62
10	34	Male	33.57	2	0	322	4.58
11	36	Male	26.00	1	0	202	2.40
12	38	Male	34.86	1	0	206	1.96
13	42	Male	26.29	2	0	92	2.10
14	28	Female	34.86	1	0	178	0.81
15	37	Female	26.00	3	2	99	3.46
16	26	Male	26.71	1	0	106	1.11
17	35	Female	27.43	2	1	194	3.12
18	41	Male	35.86	2	1	188	0.96
19	28	Male	27.14	1	0	155	0.68
$N = 19$, mean $\pm$ SD	34.26 $\pm$ 4.43		29.98 $\pm$ 4.31			163 $\pm$ 57	1.89 $\pm$ 1.20

PBMC, peripheral blood mononuclear cell.

1,323,936 $\pm$ 162,814. No $RPP30^+SRY^+$ droplets were detected in any of the 10 maternal peripheral blood samples corresponding to female fetuses (Figure 4A). On the other hand, $RPP30^+SRY^+$ droplets were detected in the three samples associated with male fetuses (Figure 4B). These droplets were considered to reflect the genomic information of male fetal cells in maternal blood circulation.

Discussion

Various types of single-cell analysis systems with high efficiency and precise reproducibility have been developed owing to improvements in microfluidics, enabling nanoliter-level reactions that provide genomic information from an extremely small volume of a targeted sample. In particular, these systems have dramatically improved single-cell RNA sequencing for various research applications, enabling the determination of gene expression status in each analyzable cell.[24–27] These investigations have provided insight into cell-development processes that cannot be obtained through bulk-cell analyses in which gene expression is averaged.[27,28] However, there are only two copies of genomic DNA in each cell. Therefore, single-cell DNA research with these systems has been limited. To increase the amount of DNA, several systems have applied whole-genome amplification steps, which result in biased amplification of genomic regions.[28,29] The sc-ddPCR system is able to directly extract targeted genomic DNA from each analyzable cell without whole-genome amplification. Moreover, single-cell analysis systems, including our sc-ddPCR system, can assess numerous cells simultaneously, although there are differences in the simultaneously adaptable or analyzable cell numbers in each system. The current sc-ddPCR system is advantageous in this respect compared with other systems; it can simultaneously analyze up to 3000 cells per well in droplet form, and 20% to 30% of all adapted cells can be individually analyzed.[23] Moreover, our modification of the system for use with cruder cell suspensions did not compromise the high sensitivity and analyzable cell rate. The results demonstrated that this sc-ddPCR system can be adapted for other biological samples that contain various impurities and can analyze single-cell genomic DNA from rare cells in such samples.

Using our modified sc-ddPCR system with high sensitivity, extremely rare male fetal cells were detected in the maternal blood samples corresponding to male fetuses. However, $RPP30^+SRY^+$ droplets were not detected among a total of 13,282,387 droplets that were generated from 10 maternal blood samples associated with female fetuses, revealing that our modified sc-ddPCR system had not only high sensitivity but also high specificity. This high specificity supports the reliability of $RPP30^+SRY^+$ droplets as a representation of genomic DNA from circulating fetal cells. To verify that circulating fetal cells could be genetically assessed using our sc-ddPCR system, we selected the *SRY* gene as a target. Our results demonstrated that other target genes can be assessed using the sc-ddPCR system. Furthermore, not only copy numbers but also single-nucleotide alterations may be technically assessed at a single-cell level with this sc-ddPCR system.

Table 3 Summary of the Genetic Assessment of Circulating Fetal Cells from 10-mL Maternal Peripheral Blood Samples Using Our Modified Single-Cell–Based Droplet Digital PCR System

Sample no.	Maternal age, years	Gestational age at blood sampling, weeks	Fetal sex on ultrasonography	Gravida	Parity	Total droplet counts	$SRY^{+}RPP30^{+}$ droplet	Neonatal external genital	Neonatal *SRY* confirmation
20	36	32.57	Female	2	1	1,208,023	−	Female	No sample
21	31	26.57	Female	1	0	1,073,928	−	Female	−
22	39	26.29	Female	2	1	1,421,886	−	Female	−
23	32	23.14	Female	2	1	1,003,626	−	Female	No sample
24	31	28.57	Male	1	0	1,310,491	+	Male	+
25	30	28.71	Female	4	1	1,387,290	−	Female	−
26	42	28.14	Male	1	0	1,259,037	+	Male	+
27	36	28.00	Male	1	0	1,359,254	+	Male	+
28	40	36.29	Female	1	0	1,276,941	−	Female	−
29	39	34.29	Female	2	0	1,555,454	−	Female	−
30	38	24.14	Female	2	1	1,458,197	−	Female	−
31	40	25.14	Female	3	1	1,360,665	−	Female	−
32	40	33.14	Female	1	0	1,536,377	−	Female	−
$N = 13$, mean ± SD	36.5 ± 4.1	28.85 ± 4.07				1,323,936 ± 162,814			

Our modification of the sc-ddPCR system enabled the assessment of rare circulating fetal cells in the peripheral blood samples of pregnant women carrying male fetuses. However, the observation of more $RPP30^{+}SRY^{+}$ droplets in each sample was expected because two to six circulating fetal cells had been contained in 1 mL of maternal blood.[18] Novel detergents or oils for droplet preparation are needed to increase the number of analyzable cells and improve the sensitivity and specificity of this sc-ddPCR system. In this study, we collected the $CD45^{-}CD14^{-}$ cell fraction from peripheral blood samples from pregnant women in the second or third trimester by manual MACS, yielding relatively uniformly sized cells containing a few circulating fetal cells for application in our microfluidic system. To apply this modified sc-ddPCR system to earlier stages of pregnancy, the abundance of circulating fetal cells in the purified cell fraction must be increased via improvement of the purification step. However, it needs to be recognized for the further improvement of the purification step that there had been cases with remaining fetal white blood cells in the maternal body for years.[30,31] Nonetheless, the analyzable genomic information was limited to only two genomic regions because of technical limitations of signal reading in each droplet. Simultaneous reading of more fluorochrome signals in each droplet while maintaining high sensitivity and specificity may allow the implementation of this sc-ddPCR system in applied research.

In this research, we demonstrated that the modified sc-ddPCR system was able to effectively assess the genomic DNA of each targeted cell in a crudely sorted biological sample. To our knowledge, this system is unprecedented in that the DNA of numerous individual live cells can be

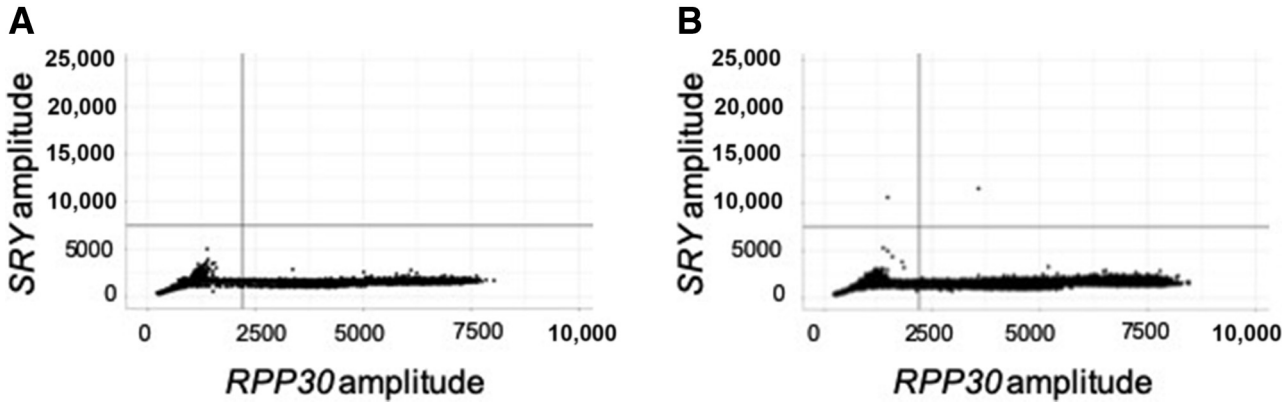

Figure 4 Determination of $RPP30^{+}SRY^{+}$ cells in maternal peripheral blood samples using the modified single-cell–based droplet digital (sc-dd) PCR system. **A** and **B:** Representative two-dimensional plots of signal amplitude of *SRY* and *RPP30* probes in each droplet prepared from a maternal peripheral blood sample corresponding to (**A**) a female fetus (number 22 in Table 3) and (**B**) a male fetus (number 24 in Table 3). The **bold lines** show thresholds for amplification of *SRY* and *RPP30*, which were determined using control samples.

simply analyzed with high sensitivity and specificity without any whole-genome amplification, cell-fixation, and cell-staining steps. Moreover, this study serves as a proof of concept for noninvasive prenatal definitive diagnosis with extremely rare circulating fetal cells using our modified sc-ddPCR system. In the future, by optimizing cell sorting and encapsulation as well as generating a more effective PCR environment in each droplet, this modified sc-ddPCR system may be a breakthrough analysis method that can be applied to various research realms, and possibly to clinical diagnostic testing.

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Author Contributions

T.S. and K.H. conceived and designed the study; T.S., O.S., and H.A. collected samples with informed consent; T.S. and Y.I. verified cell sorting; T.S., T.U., and K.H. analyzed and interpreted the data obtained using the single-cell-based droplet digital PCR system; T.S. drafted and critically revised the manuscript with support from A.O. and K.H; and all authors approved the final version of the manuscript to be published.

References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS: Presence of fetal DNA in maternal plasma and serum. Lancet 1997, 350:485–487
2. Chiu RW, Akolekar R, Zheng YW, Leung TY, Sun H, Chan KC, Lun FM, Go AT, Lau ET, To WW, Leung WC, Tang RY, Au-Yeung SK, Lam H, Kung YY, Zhang X, van Vugt JM, Minekawa R, Tang MH, Wang J, Oudejans CB, Lau TK, Nicolaides KH, Lo YM: Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. BMJ 2011, 342:c7401
3. Zimmermann B, Hill M, Gemelos G, Demko Z, Banjevic M, Baner J, Ryan A, Sigurjonsson S, Chopra N, Dodd M, Levy B, Rabinowitz M: Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. Prenat Diagn 2012, 32:1233–1241
4. Samura O, Sekizawa A, Suzumori N, Sasaki A, Wada S, Hamanoue H, et al: Current status of non-invasive prenatal testing in Japan. J Obstet Gynaecol Res 2017, 43:1245–1255
5. Lau TK, Jiang FM, Stevenson RJ, Lo TK, Chan LW, Chan MK, Lo PS, Wang W, Zhang HY, Chen F, Choy KW: Secondary findings from non-invasive prenatal testing for common fetal aneuploidies by whole genome sequencing as a clinical service. Prenat Diagn 2013, 33: 602–608
6. Sato T, Samura O, Matsuoka T, Yoshida M, Aoki H, Migita O, Okamoto A, Hata K: Molecular genetic analysis reveals atypical confined placental mosaicism with a small supernumerary marker chromosome derived from chromosome 18: a clinical report of discordant results from three prenatal tests. Eur J Med Genet 2019, 62:103533
7. Osborne CM, Hardisty E, Devers P, Kaiser-Rogers K, Hayden MA, Goodnight W, Vora NL: Discordant noninvasive prenatal testing results in a patient subsequently diagnosed with metastatic disease. Prenat Diagn 2013, 33:609–611
8. Lapaire O, Holzgreve W, Oosterwijk JC, Brinkhaus R, Bianchi DW: Georg Schmorl on trophoblasts in the maternal circulation. Placenta 2007, 28:1–5
9. Walknowska J, Conte FA, Grumbach MM: Practical and theoretical implications of fetal-maternal lymphocyte transfer. Lancet 1969, 1: 1119–1122
10. Babochkina T, Mergenthaler S, Lapaire O, Kiefer V, Yura H, Koike K, Holzgreve W, Hahn S: Evaluation of a soybean lectin-based method for the enrichment of erythroblasts. J Histochem Cytochem 2005, 53:329–330
11. Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA: Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci U S A 1990, 87:3279–3283
12. Byeon Y, Ki CS, Han KH: Isolation of nucleated red blood cells in maternal blood for non-invasive prenatal diagnosis. Biomed Microdevices 2015, 17:118
13. Ganshirt-Ahlert D, Burschyk M, Garritsen HS, Helmer L, Miny P, Horst J, Schneider HP, Holzgreve W: Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol 1992, 166:1350–1355
14. Huang R, Barber TA, Schmidt MA, Tompkins RG, Toner M, Bianchi DW, Kapur R, Flejter WL: A microfluidics approach for the isolation of nucleated red blood cells (NRBCs) from the peripheral blood of pregnant women. Prenat Diagn 2008, 28:892–899
15. Kitagawa M, Sugiura K, Omi H, Akiyama Y, Kanayama K, Shinya M, Tanaka T, Yura H, Sago H: New technique using galactose-specific lectin for isolation of fetal cells from maternal blood. Prenat Diagn 2002, 22:17–21
16. Oosterwijk JC, Knepfle CF, Mesker WE, Vrolijk H, Sloos WC, Pattenier H, Ravkin I, van Ommen GJ, Kanhai HH, Tanke HJ: Strategies for rare-event detection: an approach for automated fetal cell detection in maternal blood. Am J Hum Genet 1998, 63:1783–1792
17. Takabayashi H, Kuwabara S, Ukita T, Ikawa K, Yamafuji K, Igarashi T: Development of non-invasive fetal DNA diagnosis from maternal blood. Prenat Diagn 1995, 15:74–77
18. Krabchi K, Gros-Louis F, Yan J, Bronsard M, Masse J, Forest JC, Drouin R: Quantification of all fetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. Clin Genet 2001, 60:145–150
19. Bianchi DW, Simpson JL, Jackson LG, Elias S, Holzgreve W, Evans MI, Dukes KA, Sullivan LM, Klinger KW, Bischoff FZ, Hahn S, Johnson KL, Lewis D, Wapner RJ, de la Cruz F: Fetal sex and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. Prenat Diagn 2002, 22:609–615
20. Igarashi Y, Uchiyama T, Minegishi T, Takahashi S, Watanabe N, Kawai T, Yamada M, Ariga T, Onodera M: Single cell-based vector tracing in patients with ADA-SCID treated with stem cell gene therapy. Mol Ther Methods Clin Dev 2017, 6:8–16
21. Samura O, Sekizawa A, Zhen DK, Falco VM, Bianchi DW: Comparison of fetal cell recovery from maternal blood using a high density gradient for the initial separation step: 1.090 versus 1.119 g/ml. Prenat Diagn 2000, 20:281–286
22. Iwakawa M, Goto M, Noda S, Sagara M, Yamada S, Yamamoto N, Kawakami Y, Matsui Y, Miyazawa Y, Yamazaki H, Tsuji H, Ohno T, Mizoe J, Tsujii H, Imai T: DNA repair capacity measured by high throughput alkaline comet assays in EBV-transformed cell lines and peripheral blood cells from cancer patients and healthy volunteers. Mutat Res 2005, 588:1–6
23. Sekizawa A, Samura O, Zhen DK, Falco V, Farina A, Bianchi DW: Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. Prenat Diagn 2000, 20:886–889

24. Mora-Castilla S, To C, Vaezeslami S, Morey R, Srinivasan S, Dumdie JN, Cook-Andersen H, Jenkins J, Laurent LC: Miniaturization technologies for efficient single-cell library preparation for next-generation sequencing. J Lab Autom 2016, 21:557–567
25. Tsang JCH, Vong JSL, Ji L, Poon LCY, Jiang P, Lui KO, Ni YB, To KF, Cheng YKY, Chiu RWK, Lo YMD: Integrative single-cell and cell-free plasma RNA transcriptomics elucidates placental cellular dynamics. Proc Natl Acad Sci U S A 2017, 114: E7786–E7795
26. Zheng GX, Terry JM, Belgrader P, Ryvkin P, Bent ZW, Wilson R, Ziraldo SB, Wheeler TD, McDermott GP, Zhu J, Gregory MT, Shuga J, Montesclaros L, Underwood JG, Masquelier DA, Nishimura SY, Schnall-Levin M, Wyatt PW, Hindson CM, Bharadwaj R, Wong A, Ness KD, Beppu LW, Deeg HJ, McFarland C, Loeb KR, Valente WJ, Ericson NG, Stevens EA, Radich JP, Mikkelsen TS, Hindson BJ, Bielas JH: Massively parallel digital transcriptional profiling of single cells. Nat Commun 2017, 8:14049
27. Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS, Rinn JL: The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nat Biotechnol 2014, 32:381–386
28. Gawad C, Koh W, Quake SR: Dissecting the clonal origins of childhood acute lymphoblastic leukemia by single-cell genomics. Proc Natl Acad Sci U S A 2014, 111:17947–17952
29. Hou Y, Guo H, Cao C, Li X, Hu B, Zhu P, Wu X, Wen L, Tang F, Huang Y, Peng J: Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. Cell Res 2016, 26:304–319
30. Schroder J, Tiilikainen A, De la Chapelle A: Fetal leukocytes in the maternal circulation after delivery. I. Cytological aspects. Transplantation 1974, 17:346–354
31. Tiilikainen A, Schroder J, De la Chapelle A: Fetal leukocytes in the maternal circulation after delivery. II. Masking of HL-A antigens. Transplantation 1974, 17:355–360