High resolution array-CGH analysis of single cells

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ABSTRACT

Heterogeneity in the genome copy number of tissues is of particular importance in solid tumor biology. Furthermore, many clinical applications such as pre-implantation and non-invasive prenatal diagnosis would benefit from the ability to characterize individual single cells. As the amount of DNA from single cells is so small, several PCR protocols have been developed in an attempt to achieve unbiased amplification. Many of these approaches are suitable for subsequent cytogenetic analyses using conventional methodologies such as comparative genomic hybridization (CGH) to metaphase spreads. However, attempts to harness array-CGH for single-cell analysis to provide improved resolution have been disappointing. Here we describe a strategy that combines single-cell amplification using GenomePlex library technology (Genome-Plex® Single Cell Whole Genome Amplification Kit, Sigma-Aldrich, UK) and detailed analysis of genomic copy number changes by high-resolution array-CGH. We show that single copy changes as small as 8.3 Mb in single cells are detected reliably with single cells derived from various tumor cell lines as well as patients presenting with trisomy 21 and Prader–Willi syndrome. Our results demonstrate the potential of this technology for studies of tumor biology and for clinical diagnostics.

INTRODUCTION

Clinical applications such as pre-implantation and non-invasive prenatal diagnosis and studies of tumor heterogeneity and of single disseminated tumor cells (micrometastases) require the analysis of genome copy number changes at the single-cell level. Although this can be achieved using fluorescence *in situ* hybridization (FISH), resolution and scope are severely limited and the procedure is time consuming. Alternatively, the whole genome can be scanned for changes using comparative genomic hybridization (CGH), but as insufficient DNA is available from single cells directly for hybridization, PCR amplification protocols have been investigated for this purpose. Initially, degenerate oligonucleotide primed (DOP)-PCR (1) was used successfully by various groups to analyze copy number changes in minute subregions of specimens derived from, e.g. frozen tumor sections, formalin fixed or paraffin-embedded materials (2–5). However, the amplification of single cells compared with several hundreds of cells from tissue sections is much more demanding as unbiased amplification from such a small amount of starting DNA has proved particularly challenging.

Protocols based on linker-adaptor PCR (6) or modified DOP-PCR amplification (7,8) have been developed which yield reproducible amplification products from single cells suitable for subsequent CGH analysis onto metaphase spreads. These methods have been applied to prenatal diagnostics (9,10) and for the analyses of minimal residual disease (11,12). However, amplification of single cells using these approaches is labor-intensive and not amenable for high-throughput applications. Recently, Le Caignec *et al.* (13) reported the amplification of single lymphoblastoid cells, fibroblasts and blastomeres by use of multiple displacement amplification (MDA) and subsequent copy number analysis by array-CGH. This approach was able to detect aneuploidies of whole chromosomes, however, with a detection resolution of 34 Mb at best, it failed to demonstrate a significant improvement compared with conventional methodologies.

Here we describe a strategy combining single-cell amplification by use of GenomePlex library technology (GenomePlex® Single Cell Whole Genome Amplification Kit, Sigma-Aldrich, UK) and genomic copy number analysis employing high-resolution array-CGH (14). The GenomePlex library technology allows a representative amplification of genomic DNA based on random fragmentation and subsequent conversion to PCR amplifiable products. Using this approach, we have

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been able to detect copy number changes as small as 8.3 Mb in microdissected tumor cells and cells derived from patients with trisomy 21 and Prader–Willi sydrome.

MATERIALS AND METHODS

Patient samples/cell lines

Genomic DNA derived from patients with trisomy 21 and Prader–Willi Syndrome was isolated from peripheral blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the suppliers. The Chromosome 15q11–13 microdeletion was confirmed by FISH onto metaphase spreads of the patient, using a commercially available FISH probe mapping to the SNRPN-gene (Abbott-Vysis).

The primary renal cell adenocarcinoma line (769P, ATCC No. CRL-1933) and the colorectal cell line HCT116 (generously provided by Dr Christoph Lengauer and Dr Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) were grown in McCoy's 5A medium (Gibco Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Genomic DNA was isolated as described above.

The karyotype of HCT116 has previously been described as 45, X–Y, der (10) dup (10) (q24q26)t(10;16)(q26;q24), der (14) t(8;16)(q13;p13), der (15) t(17;18)(q21;p11.3) (15)

Preparation of cells on membrane-coated slides

Cell suspensions were prepared for microdissection by centrifuging the cells for 10 min at 120 *g*. Pellets were washed twice with phosphate-buffered saline (PBS) and resuspended to obtain ∼2.5 × 10^6 cells/ml. 200 μl of the cell suspension was then transferred by cytocentrifugation (120 *g* for 3 min) onto slides covered with a polyethylene-naphthalate (PEN) membrane (PALM Microlaser Technologies, Bernried, Germany).

Isolation of cells by laser microdissection and pressure catapulting

Single cells were isolated using the PALM MicroBeam System (PALM Microlaser Technologies, Bernried, Germany) as described previously (11,16,17) and collected in a 200 μl Eppendorf tube cap containing 10 μl lysis and fragmentation buffer as detailed below.

Single-cell amplification

Single-cell amplification was performed using the GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, UK) according to the instructions of the suppliers but with slight modification. Briefly, after catapulting single cells into Eppendorf caps containing a mix of 1 μl lysis and fragmentation buffer/proteinase K solution and 9 μl of water, individual cells were collected in separate tubes by centrifugation for 10 min at 18 890 *g*. Cell lysis, fragmentation and library preparation was performed according to the instructions supplied in the kit. Amplification of the library was performed as follows: 7.5 μl of 10× Amplification Master Mix, 51 μl of nuclease free water and 1.5 μl Titanium *Taq* DNA polymerase (BD Biosciences Clontech, Heidelberg, Germany) was added to 14 μl library mix. Samples

were amplified using an initial denaturation of 95°C for 3 min followed by 23 cycles each consisting of a denaturation step at 94°C for 15 s and an annealing/extension step at 65°C for 5 min. The reaction mixture was stored at −20°C until further use.

Array-CGH

Whole genome tiling path arrays employed in this study were prepared as described previously (14). The clone set used for array construction was verified by fingerprinting and BAC end sequencing, and can be viewed within the Ensembl genome browser (http://www.ensembl.org/*Homo_sapiens*/cytoview). Arrays were printed at the UCSF microarray facility (http://cc.ucsf.edu/microarray) and stored at room temperature until use.

Array-CGH was performed as described previously with slight modifications (14). Briefly, 200 ng of single-cell amplification products and non-amplified genomic male reference DNA were differentially labeled with dCTP-Cy3 or dCTP-Cy5 (NEN Life Science Products) in a 150 μl reaction using a Bioprime Labeling Kit (Invitrogen, Carlsberg, CA, USA). All experiments were performed at least as duplicate, color reversal hybridizations. Unincorporated nucleotides were removed by use of Microcon YM-30 filter devices (Millipore Co.) according to the instructions of the suppliers.

Hybridizations were carried out by use of a Tecan HS™ Hybridization Station (Tecan Group Ltd.). For hybridization to an array of an area of 1.8 × 3.6 cm, Cy3 and Cy5 labeled DNAs were combined, precipitated with 135 μg of human Cot1 DNA (Roche Diagnostics Ltd, UK) and 33 μg of *E.coli* genomic DNA (InvivoGen, Toulouse, France) and resuspended in 120 μl of hybridization buffer (50% formamide, 5% dextran sulfate, 0.1% Tween-20, 2× SSC, 10 mM Tris–Hcl, pH 7.4 and 10 mM cysteamine). Pre-hybridization solution was prepared simultaneously by precipitating 100 μl of herring sperm DNA (10 mg/ml; Sigma-Aldrich, UK) and 33 μg of *E.coli* genomic DNA (InvivoGen, Toulouse, France) and resuspending in 165 μl of hybridization buffer.

The pre-hybridization and hybridization solutions were then denatured for 10 min at 72°C. Into the hybridization station chamber, 100 μl of the pre-hybridization mix was then injected following instructions displayed on the station. During pre-hybridization (45 min at 37°C), the hybridization mix was incubated at 37°C. Hybridizations were carried out for 45 h at 37°C with medium agitation frequency. Slides were washed with PBS/0.05% Tween-20/2 mM cysteamine (wash time 0.5 min, soak time 0.5 min, 15 cycles at 37°C), 0.1× SSC (wash time 1.0 min, soak time 2.0 min, 5 cycles at 54°C), PBS/0.05% Tween 20/2 mM cysteamine (wash time 0.5 min, soak time 0.5 min, 10 cycles at 23°C) and HPLC water (wash time 0.5 min, soak time 0.0 min, 1 cycle at 23°C) before drying for 2.5 min using nitrogen gas.

Data analysis

The arrays were scanned using an Agilent scanner (Agilent Technologies, UK) and images quantified by use of GenePix version 6.0 software (GRI, UK). For each individual hybridization, the fluorescent ratios were normalized by dividing the raw ratios of each clone by the median ratio of all autosomal clones present on the array. Replicate experiments were

combined and data points were accepted if they were within ±10% of the median and if at least two accepted data points remained. For comparison with Le Caignec *et al*. (13), a subset of clones spaced at ∼1 Mb intervals across all chromosomes was selected from the full tile path set and this reduced resolution analysis applied to hybridizations with the trisomy 21 and Prader–Willi cases. The clone selection was made by simply identifying the clone mapping closest to the start of each 1 Mb interval of each chromosome. The average clone spacing of this subset was 1.03 Mb. For full tiling path analysis, the average ratio across 10 clones was calculated for each chromosome and plotted against the midpoint position of the 10 clones used for analysis. Each averaged data point was then normalized further by dividing by the median ratio of all autosomal averaged data points.

The estimate of experimental variability (SDe) was calculated using the 68.2th percentile value of absolute dye-swap combined ratios as described in Fiegler *et al*. (14) for DNA from the microdeletion and trisomy 21 cases. In a normal distribution, the area within ±1 SD from the mean contains 68.2% of all values. The 68.2th percentile value thus provides an estimation of the standard deviation, which is relatively insensitive to outlying values.

However, for DNA from tumor cell lines, copy number changes (outliers) often make up >31.8% of ratio values such that the 68.2% is no longer a good estimate of the variance of the central distribution. Therefore, for the two tumor cell lines we used the 34.1% value which defines ±0.46 SD, scaling this value accordingly. Significant gains and loses were defined as regions of at least three consecutive data points all above or all below 1.5 times the SDe.

Raw normalized ratios for all arrays, mean ratios across 10 tilepath clones and data for the 1 Mb selection can be found in Supplementary data files 1, 2 and 3.

RESULTS

Three cells each of two different tumor cell lines and patients with defined constitutional rearrangements were microdissected, independently treated and amplified using the GenomePlex library technology. The Single Cell WGA Kit produced a consistent yield (between 3 and 6 µg) and size range as visualized by agarose gel electrophoresis (Figure 1). The amplification products of each cell were then used for analysis by high-resolution array-CGH. The array used in this study consists of 26 574 clones selected from the published Golden Path and covers 93.7% of euchromatic regions in tiling path resolution. This array has been employed to survey copy number variations in the human genome and has been subjected to high levels of validation (14,18). Hybridizations of single-cell amplifications generally showed an increase in variation compared with corresponding non-amplified DNA hybridizations. However, hybridizations were consistent as the mean difference in the standard deviation between duplicate dye-swap experiments (*n* = 6) was <6%.

Hybridization with a renal cell carcinoma cell line (769P)

To test whether single copy number changes of large regions could be detected using single-cell array-CGH, we compared
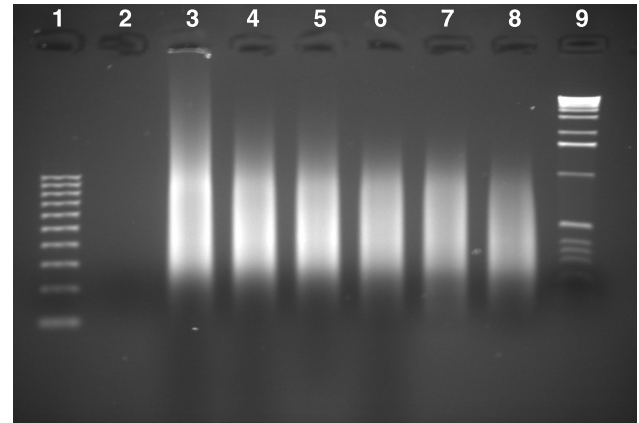

Figure 1. Gel image of amplified single cells. lane 1: 100 kb size marker; lane 2: negative control; lane 3: positive control provided in the Sigma kit; lane 4: single cell—normal female; lane 5: single cell—trisomy 21; lane 6: single cell—HCT116; lane 7: single cell—Prader–Willi syndrome; lane 8: single cell—769P; lane 9: lambda-HindIII size marker.

hybridizations of single-cell amplification products and non-amplified genomic DNA of a female renal cell carcinoma cell line (769P). This cell line in our laboratory has been studied extensively by M-FISH and array-CGH (14,19) and is primarily tetraploid but with the equivalent of single copy gains and losses (relative to diploid state) across the genome. In general, areas of copy number change identified by hybridization of non-amplified DNA could be detected with the single-cell products. Chromosome 1, e.g. harbors the equivalent of a single copy deletion on the p-arm covering a region of ∼30 Mb and the equivalent of a single copy gain on the q-arm of ∼90 Mb. Both regions of copy number change were identified in single-cell amplified material and non-amplified DNA (Figure 2). Mean ratios across these regions on Chromosome 1 compared well between the two applications, however, standard deviations were generally increased when hybridizing single-cell amplification products (Table 1). Moreover, standard deviations across all autosomes were significantly increased when hybridizing amplified single-cell material [SDe (single-cell products): 0.24 (Cell 1), 0.27 (Cell 2) and 0.22 (Cell 3), SDe (non-amplified DNA):0.07].

Hybridization with HCT116

Hybridization of a colorectal tumor cell line, HCT 116, produced similar observations. Experimental variability (SDe) was 0.18 (Cell 1), 0.19 (Cell 2) and 0.26 (Cell 3) across the autosomes for single cells, compared with 0.05 for non-amplified genomic DNA. Moreover, we identified small differences between amplified single-cell DNA and non-amplified genomic DNA and within single-cell amplifications. For example, we identified a 8.3 Mb single copy deletion on Chromosome 3 (genomic position 0.7–9.0 Mb) followed by a gain of 9.2 Mb (genomic position 18.4–27.6 Mb) in one out of the three cells analyzed, which was also not present in non-amplified DNA (Figure 3).

Hybridization of patient DNA

In order to test whether this amplification method is also suitable for the types of analyses required for prenatal diagnostic
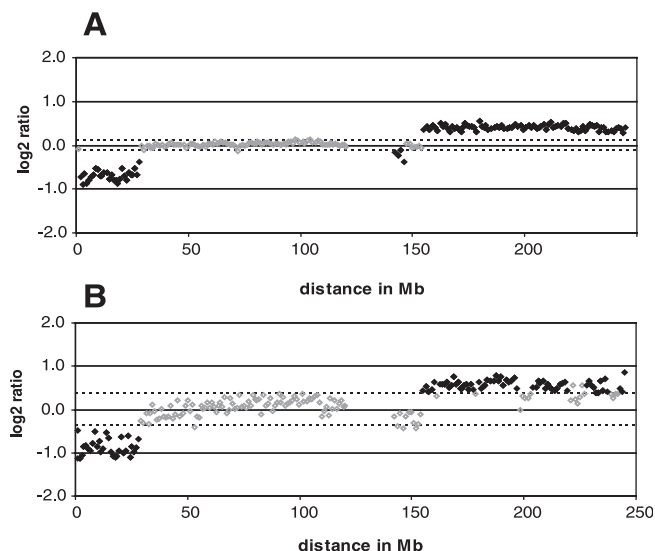
Figure 2. Array-CGH analysis of a female renal cell carcinoma cell line (769P) **(A)**. Chromosome 1 profile using non-amplified genomic DNA *versus* non-amplified genomic DNA of a normal male individual (threshold ± 0.11). **(B)**. Chromosome 1 profile, using amplified single-cell DNA, versus non-amplified genomic DNA of a normal male individual (threshold ±0.37). Closed diamonds: data points called above or below a threshold of 1.5× SDe indicating areas of single copy gain or loss detected across the chromosome. Open diamonds: non-called data points. Thresholds are indicated by black dashed lines.

Table 1. The mean log2 ratios and corresponding SD across modal regions and areas of single copy number gain and loss across Chromosome 1 for single cell amplifications ($n = 3$) and non-amplified genomic DNA

Region per 10 clones (in Mb)	Mean log2 ratio	SD
1.2–29.3	sc-products: −0.87/−0.63/−0.68	sc-products: 0.19/0.27/0.23
	genomic DNA: −0.58	genomic DNA: 0.15
29.3–155.1	sc-products: 0.04 /0.07/0.10	sc-products: 0.19/0.19/0.20
	genomic DNA: 0.006	genomic DNA: 0.07
155.1–244.9	sc-products: 0.51/ 0.61/0.54	sc-products: 0.15/0.13/0.17
	genomic DNA: 0.4	genomic DNA: 0.06

tests, we hybridized amplified single-cell material derived from a female patient diagnosed with trisomy 21 and a male patient with an established microdeletion (Prader–Willi syndrome) both against non-amplified male control DNA. CGH analysis using single-cell amplification products of the trisomy 21 patient revealed a ratio increase affecting nearly all Chromosome 21 data points in all three cells analyzed, with 22/28 data points (Cell 1), 23/28 data points (Cell 2) and 19/28 data points (Cell 3) reporting the trisomy (Figure 4A–C). Additional small regions of gain or loss were reported on various other chromosomes. Unfortunately, we could not obtain genomic DNA from this patient for comparison and so cannot confirm these observations in non-amplified DNA.

This same data set was reanalyzed at a simulated resolution of 1 clone per Mb for comparison with the study of Le Caignec *et al.* (13). While all Chromosome 21 data points showed an increased ratio, due to the higher level of variation, fewer data points were called according to our criteria
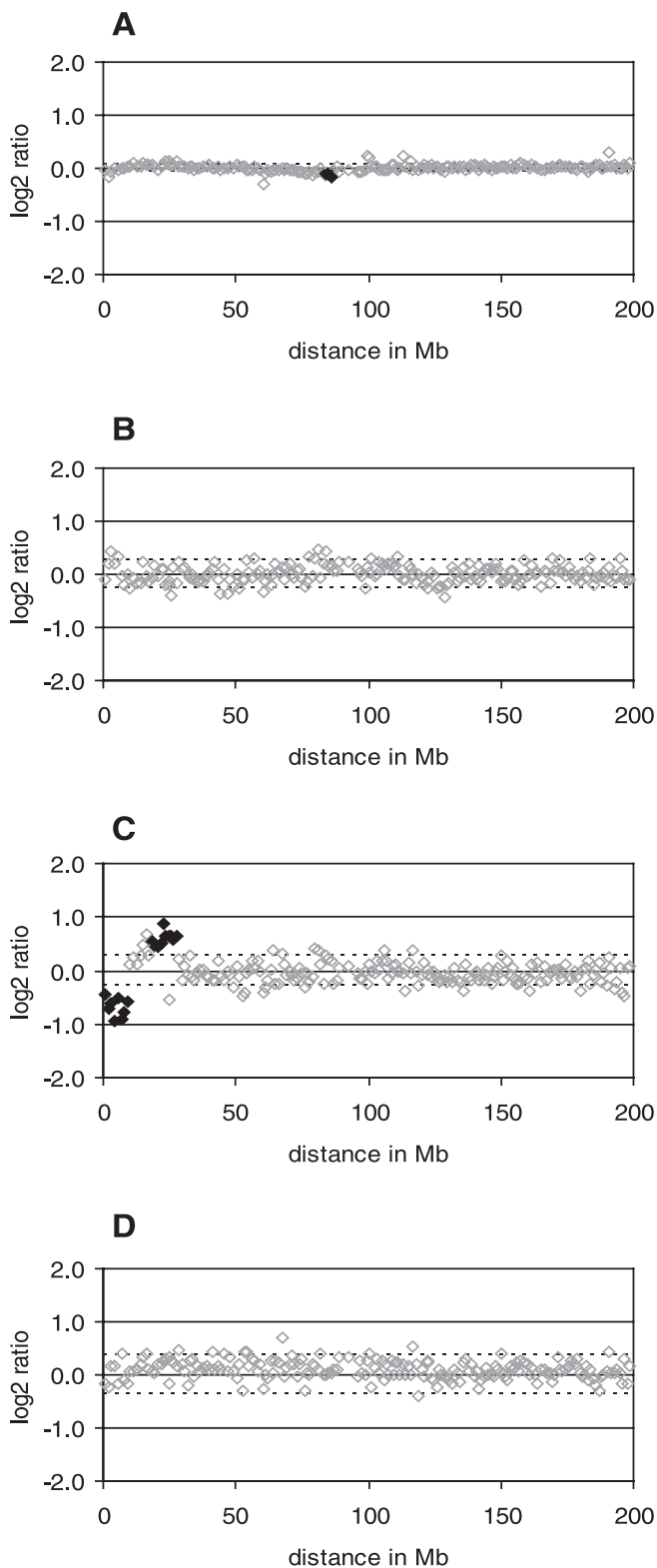

Figure 3. Array-CGH analysis of a male colorectal cell line (HCT116). Chromosome 3 profiles of non-amplified control DNA **(A)** (threshold ± 0.081) and three independently isolated and treated single cells [thresholds ± 0.266 (Cell 1; **B**), ±0.291 (Cell 2; **C**) and ±0.381 (Cell 3; **D**)]. Closed diamonds: data points called above or below a threshold of 1.5× SDe indicating areas of single copy gain or loss detected across the chromosome. Open diamonds: non-called data points. Thresholds are indicated by black dashed lines.
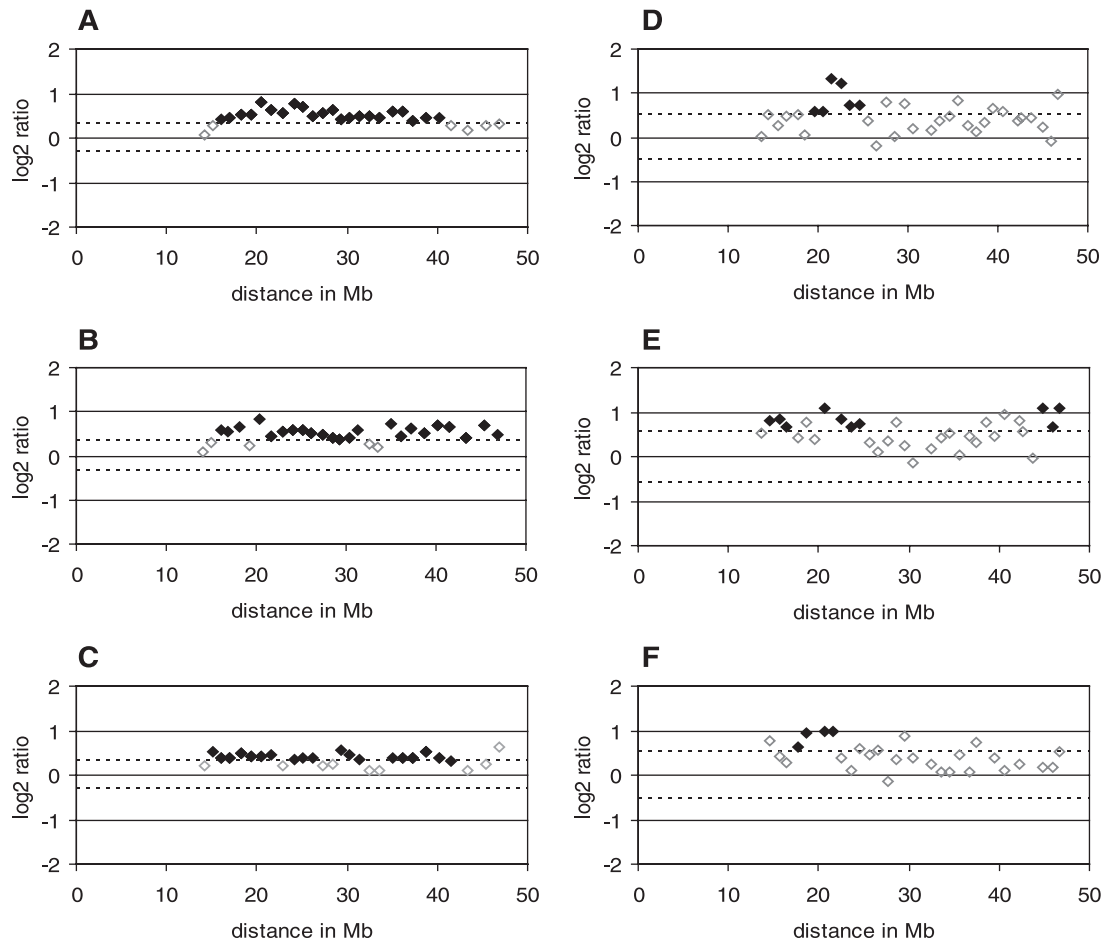
Figure 4. Array-CGH analysis of a female patient presenting with Down syndrome. Chromosome 21 profiles of three independently isolated and treated cells using the full tiling path set with thresholds of ±0.305 (Cell 1; **A**), ±0.35 (Cell 2; **B**) and ±0.308 (Cell 3; **C**) and the simulated 1 Mb data set with thresholds of ±0.52 (Cell 1; **D**), ±0.57 (Cell 2; **E**) and ±0.53 (Cell 3; **F**). Closed diamonds: data points called above or below a threshold of 1.5× SDe indicating areas of single copy gain or loss detected across the chromosome. Open diamonds: non-called data points. Thresholds are indicated by black dashed lines.

for significant regions of gain of loss (Figure 4 D–F). However, using the same whole chromosome averaging approach as Le Caignec *et al*. (13) with a threshold for significance set at ±3 times the standard deviation of all autosomes excluding Chromosome 21, the trisomy 21 was easily detected. For each of the three cells, only Chromsome 21 showed a significant copy number change (Figure 5). Overall, the variance of the chromosome averages for data from our simulated 1 Mb resolution analysis was lower than the equivalent data from Le Caignec *et al*. (13) (Table 2). For example, the mean Chromosome 21 ratio for the three cells was 0.49 for the Le Caignec *et al*. study and 0.48 in this study but the standard deviations of the means were 0.11 and 0.07 respectively. Similarly, the standard deviation of autosome means (excluding Chromosome 21) for all cells was 0.13 for the Le Caignec *et al*. study and 0.08 in this study.

For a patient with Prader–Willi syndrome, array-CGH analysis confirmed a microdeletion, previously identified using a single FISH probe on Chromosome 15, with a size of ~10.8 Mb (Figure 6A–D). This deleted region was called in non-amplified DNA (genomic position 20.0–30.8 Mb involving 11 data points) and Cells 1 (genomic position 18.8–30.8 Mb involving 12 data points), 2 and 3 (genomic position 20.0–30.0 Mb involving 10 data points).

Again, hybridizations with single-cell amplification products were noisier [SDe of 0.14 (Cell 1), 0.17 (Cell 2) and 0.14 (Cell 3)] than the equivalent hybridization with non-amplified DNA (SDe = 0.03). With the exception of three small regions of reported copy number loss on Chromosomes 1, 11 and 20 detected in two of the three cells analyzed, none of the additional copy number changes were reported in the three hybridizations using single-cell products [12 regions involving 50 data points (Cell 1), 17 regions involving 59 data points (Cell 2) and 20 regions involving 70 data points (Cell 3)] could be verified in the control hybridization producing a false positive rate of 2.0 (Cell 1), 2.4 (Cell 2) and 2.8% (Cell 3). Conversely, when applying the same threshold parameters for the control hybridization using non-amplified genomic DNA, 23 additional regions involving 75 data points reported small but significant copy number changes which were not detected in the single-cell amplifications, producing a false negative rate of 3.0%.

Reanalysis of Chromosome 15 with the simulated 1 Mb resolution clone set showed further increase in variation particularly for the non-deleted region (Figure 6 and Table 3). Furthermore, although the deletion involving the expected region was observed in all three cells, using the 1 Mb data set fewer and a more variable number of clones reported
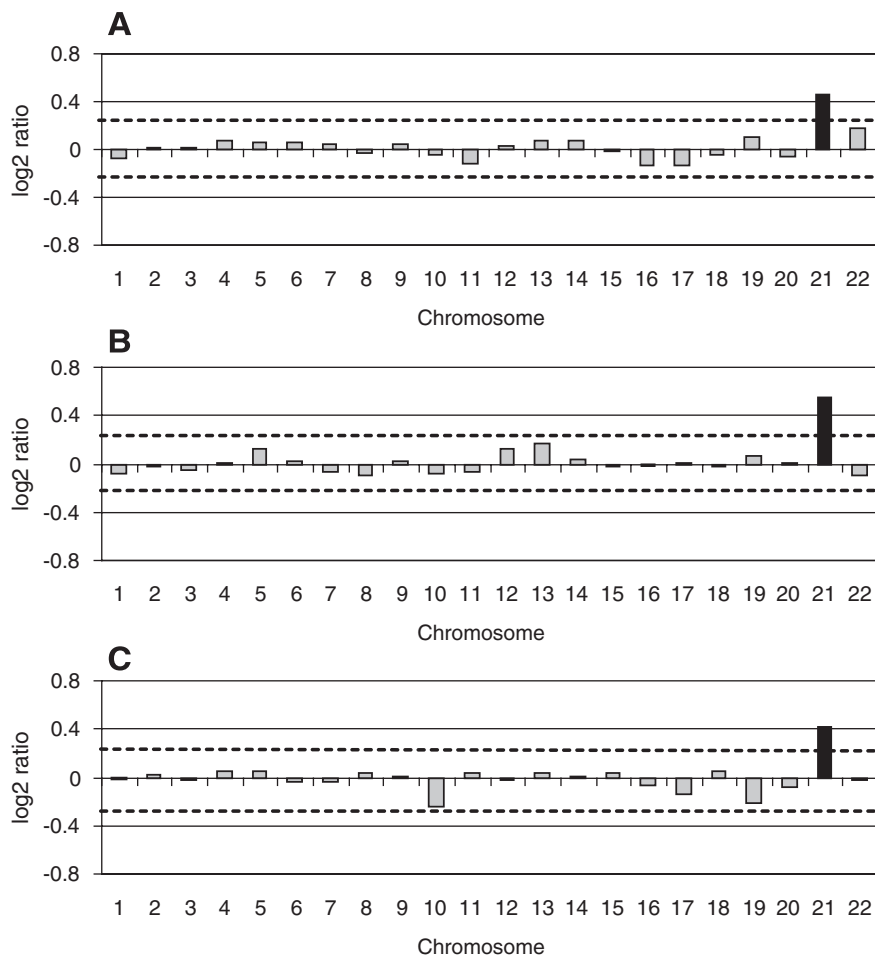
Figure 5. Log2 ratio average across autosomes in three individually treated cells derived from a patient presenting with Down syndrome. Grey bars: chromosome averages within a threshold of ±3× SD; black bars: chromosome averages above/below a threshold of ±3× SD. Thresholds are indicated by black dashed lines and have been calculated as 0.24 (Cell 1; **A**), 0.23 (Cell 2; **B**) and 0.25 (Cell 3; **C**)

the deletion [13/15 data points (Cell 1), 8/15 data points (Cell 2) and 6/15 data points (Cell 3)] (Figure 6E–H).

DISCUSSION

The detection of copy number changes in single cells is of great importance for many clinical applications, in particular pre-implantation and non-invasive prenatal diagnosis as well as studies of tumor heterogeneity and micrometastasis. Nevertheless, the unbiased amplification of the small amounts of DNA available from single cells has proved particularly challenging. Recently, Hu *et al*., (20) have used a DOP-PCR approach to amplify single lymphocytes and fibroblast cells for subsequent detection of chromosome aneuploidies by low resolution (whole chromosome) array-CGH. Although they were able to detect known whole chromosome trisomies, replicate control experiments using normal male or female single lymphocytes were highly variable such that false chromosome aneuploidies were found in many experiments. Le Caignec *et al*. (13) described a strategy that combined multiple displacement amplification (MDA) and array-CGH to determine aneuploidy levels in single lymphoblastoid cells, fibroblasts and blastomeres. Although this group used

an array with a resolution of ~1 clone every Mb across the genome, for the analysis of trisomy, clone ratios were averaged across whole chromosomes. In single trisomy 13, 18 and 21 fibroblasts, mean log2 ratios of the affected chromosomes were found to be 0.58, 0.55 and 0.49, respectively (theoretical ratio for trisomy = 0.58), while mean ratios of the unaffected chromosomes in general were close to zero. Although this approach appears to be more reproducible than the previous study, replicate experiments still showed a high degree of variability requiring smoothing or averaging of data points. While Le Caignec *et al*. (13) demonstrated that a deletion of 34 Mb could be detected by averaging all of the 39 clones within this previously identified region, they did not investigate the minimum level of smoothing required for reliable detection of copy number changes and so the absolute resolution of their strategy for detecting unknown copy number changes.

We have employed the GenomePlex library technology (GenomePlex® Single Cell Whole Genome Amplification Kit, Sigma-Aldrich, UK) for amplification of microdissected single cells derived from tumor cell lines and patients with constitutional rearrangements and characterized genomic copy number changes by high-resolution array-CGH. The detection of chromosome aneuploidies was tested by analyzing

Table 2. Averaged log2 intensity ratios across autosomes in three independently treated cells derived from a female patient presenting with trisomy 21

Chromosome	T21–1 (A) (13)	T21–2 (A) (13)	T21–3 (A) (13)	T21–1 (B) (this study) 1 Mb	T21–2 (B) (this study) 1 Mb	T21–3 (B) (this study) 1 Mb	T21–1 (C) (this study) tile path	T21–2 (C) (this study) tile path	T21–3 (C) (this study) tile path
1	0.14	0.01	0.08	−0.07	−0.08	0.00	−0.07	−0.06	−0.03
2	0.16	0.12	0.12	0.02	−0.02	0.02	0.04	0.00	0.03
3	0.18	0.12	0.14	0.01	−0.04	−0.02	0.01	−0.02	0.00
4	0.05	0.09	0.13	0.08	0.01	0.06	0.10	0.04	0.08
5	−0.04	0.09	0.25	0.05	0.13	0.05	0.02	0.09	0.06
6	0.15	0.10	0.13	0.06	0.02	−0.04	0.04	0.06	−0.02
7	−0.03	−0.01	0.07	0.05	−0.07	−0.04	0.02	−0.09	−0.03
8	0.08	0.22	0.02	−0.03	−0.10	0.04	−0.01	−0.12	0.04
9	0.12	0.09	−0.05	0.04	0.02	0.00	0.01	0.02	0.00
10	0.03	0.07	0.07	−0.04	−0.08	−0.24	0.02	−0.06	−0.16
11	0.01	−0.06	0.06	−0.12	−0.07	0.04	−0.10	−0.04	0.03
12	0.04	0.04	0.04	0.04	0.13	−0.02	0.00	0.12	−0.02
13	0.03	0.11	0.00	0.08	0.17	0.04	0.10	0.18	0.02
14	0.00	0.09	0.05	0.07	0.04	0.01	0.04	0.04	0.00
15	0.10	0.12	−0.02	−0.01	−0.02	0.03	−0.04	0.02	−0.05
16	0.16	−0.12	−0.06	−0.13	−0.01	−0.07	0.02	0.01	−0.04
17	0.06	−0.09	0.09	−0.13	0.01	−0.14	−0.12	−0.04	−0.17
18	0.29	0.07	0.23	−0.04	−0.02	0.06	0.00	0.00	0.03
19	−0.26	−0.20	−0.06	0.10	0.07	−0.22	0.07	0.04	−0.22
20	−0.21	−0.06	0.02	−0.06	0.01	−0.08	−0.09	−0.13	−0.06
21	**0.50**	**0.38**	**0.59**	**0.46**	**0.56**	**0.42**	**0.47**	**0.49**	**0.36**
22	0.12	−0.29	−0.41	0.17	−0.09	−0.03	0.15	−0.05	−0.07
Mean ± SD	**0.06±0.13**	**0.02±0.13**	**0.04±0.13**	**0.01±0.08**	**0.00±0.08**	**−0.03±0.08**	**0.01±0.07**	**0.00±0.08**	**−0.03±0.08**
Overall mean ± SD		**0.04±0.13**			**−0.01±0.08**			**−0.01±0.07**	

A: data derived from Le Caignec *et al.* (13)
B: data derived from this study and analyzed using the simulated 1 Mb set.
C: data derived from this study and analyzed using the tile path clones.
Chromosome 21 intensity ratios are highlighted in bold.

single-cell material derived from a patient presenting with trisomy 21. We found that the vast majority of data points (average of 10 clones equivalent to ~1 Mb) representing Chromosome 21 showed a single copy gain in all three individually treated cells. However, we too found increased variability associated with single-cell amplification compared with non-amplified genomic DNA. This variability as well as potential differences in DNA representation in amplifications may well account for the small number of the data points that did not reach the significance threshold. For comparison of our results with the study of Le Caignec *et al.* (13) we calculated the mean ratios across the chromosomes for all three cells (Table 2) using the simulated 1 clone per Mb data set. Although mean ratios of the non-affected autosomes were found to be similarly ~0, Chromosome 21 clones reported mean ratios of 0.46, 0.56 and 0.42 for the three cells which compares well with the results obtained by Le Caignec *et al.* who reported mean ratios of 0.38, 0.50 and 0.59, respectively. However, in general our results were less variable with a mean and SD of −0.01 ± 0.08 for the other autosomes compared with 0.04 ± 0.13 for the study of Le Caignec *et al.* This would suggest that the amplification method we have used produces less variable results than the MDA method used by Le Caignec *et al.* although these small differences could also be explained by the use of different arrays and hybridization protocols. However, the most dramatic improvement in our results is due to the possibility to maintain a 1 Mb resolution while smoothing the data by averaging 10 tiling path clones (Table 2, Figure 4).

With the development of high-resolution array-CGH, the detection of microdeletions and microduplications in patients with constitutional rearrangements as well as prenatal detection of well characterized microdeletion/microduplication syndromes has become a widespread application for this technique. We were curious to see whether our single cell approach would be suited to identify reliably such small copy number changes across the genome. We, therefore, analyzed single-cell material derived from a male patient presenting with Prader–Willi syndrome and compared the results with hybridizations using non-amplified control DNA. An unusually large single copy deletion on Chromosome 15, which had previously been confirmed using a commercially available FISH probe, was detected in all three single cells as well as in the control DNA and could be sized ~10.8 Mb. The size of this deletion is unusual as patients with Prader–Willi syndrome usually have a deletion of 4 Mb (21). Chromosome analysis excluded the presence of a complex chromosomal rearrangement and confirmed the presence of an interstitial deletion (data not shown). However, we also tested DNA from a patient with DiGeorge syndrome with a deletion of 3 Mb, which was not detected in single-cell amplifications (data not shown). This result is not unexpected as the variability of the single-cell amplifications necessitated smoothing of the data by averaging 10 clones and requiring three data points to exceed the threshold, which reduces the resolution of our strategy to ~3 Mb. The DiGeorge region is also rich in segmental duplications which are known to reduce the response of clones to specific copy number changes.
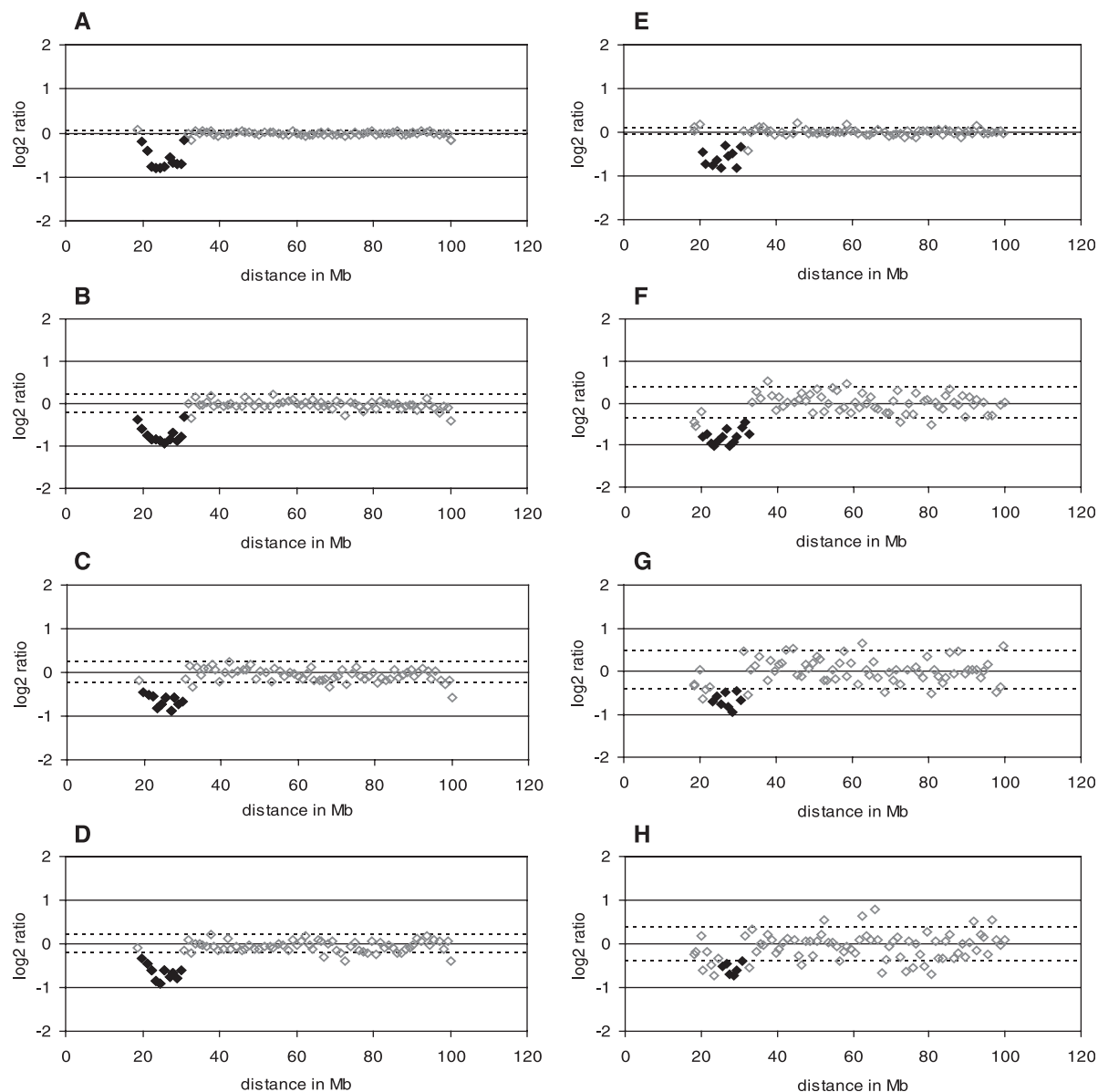
Figure 6. Array-CGH analysis of a male patient presenting with Prader Willi syndrome. Chromosome 15 profiles of non-amplified control DNA and three independently isolated and treated single cells analyzed using the full tiling path set and with thresholds of ±0.05 [non-amplified DNA (**A**)], ±0.21 (Cell 1; **B**), ±0.25 (Cell 2; **C**) and ±0.21 (Cell 3; **D**) and the simulated 1 Mb data set with thresholds of ±0.08 [non-amplified DNA (**E**)], ±0.37 (Cell 1; **F**), ±0.44 (Cell 2; **G**) and ±0.38 (Cell 3; **H**). Closed diamonds: data points called above or below a threshold of 1.5× SDe indicating areas of single copy gain or loss detected across the chromosome. Open diamonds: non-called data points. Thresholds are indicated by black dashed lines.

To test the detection limitations of our approach further, we then analyzed two different, previously well characterized tumor cell lines which are known to harbor various single copy number changes of different sizes. For example, the renal carcinoma cell line, 769P, harbors the equivalent of a single copy number loss of ∼30 Mb on Chromosome 1p and a single copy number gain of ∼90 Mb on Chromosome 1q, both of which were readily detected by our methodology. However, 769P also has a small single copy deletion on Chromosome 9 of ∼6.3 Mb (genomic position 16.7–23.0 Mb), which due to our stringent calling parameters was only called partially in one of the three single cells analyzed, although lower ratios consistent with a deletion of this region could be seen in the other two single cells (Figure 7).

We are thus able to conclude that the resolution of our strategy to detect single copy deletions in single cells is at least 10.8 Mb but not as low as 6.3 Mb.

It is well known that heterogeneity in the genomic constitution of cells within solid tumors is an important feature in the natural history of tumor progression and metastasis. Accessing the exact copy number status of individual cells or localized groups of cells within tumors has been technically challenging. We identified copy number differences between individual single cells of both tumor cell lines we studied. For example, when analyzing the amplification products derived from single HCT116 cells, we identified a 8.3 Mb single copy deletion on Chromosome 3 (genomic position 0.7–9.0 Mb) followed by a gain of 9.2 Mb (18.4–27.6 Mb)

Table 3. The mean log2 ratios and corresponding SDs across the deleted and non-deleted regions on Chromosome 15 in DNA derived from a patient with Prader–Willi syndrome

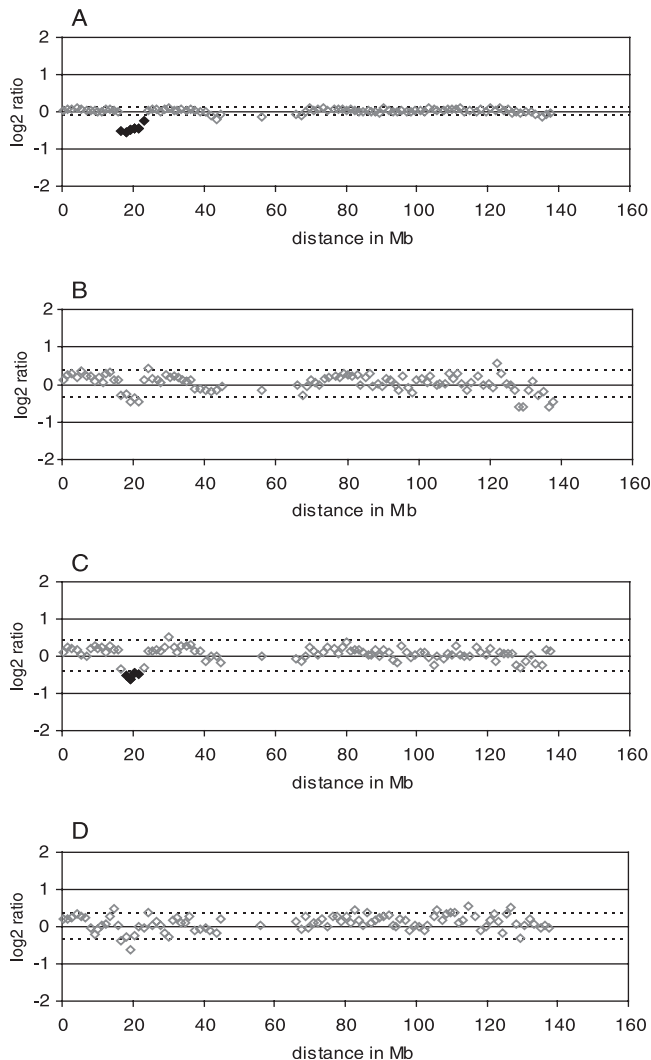|	Deleted region 1 Mb analysis	Non-deleted region 1 Mb analysis	Deleted region tile path analysis	Non-deleted region tile path analysis
Cell 1	−0.72 ± 0.23	0.01 ± 0.22	−0.65 ± 0.28	−0.02 ± 0.10
Cell 2	−0.47 ± 0.34	0.03 ± 0.27	−0.50 ± 0.29	−0.06 ± 0.14
Cell 3	−0.40 ± 0.29	−0.03 ± 0.30	−0.50 ± 0.31	−0.06 ± 0.13

Figure 7. Array-CGH analysis of a female renal cell carcinoma cell line (769P). Chromosome 9 profile of non-amplified control DNA and three independently isolated and treated single cells with thresholds of ±0.11 [non-amplified DNA (**A**)], ±0.37 (Cell 1; **B**), ±0.43 (Cell 2; **C**) and ±0.36 (Cell 3; **D**). Closed diamonds: data points called above or below a threshold of 1.5× SDe indicating areas of single copy gain or loss detected across the chromosome. Open diamonds: non-called data points. Thresholds are indicated by black dashed lines.

in one out of the three cells analyzed. HCT116 is a colorectal cell line, which has often been used because of its stable karyotype. Our single-cell array-CGH observation of an imbalance on Chromosome 3 may, therefore, represent merely a rare

finding. Alternatively, it may suggest that small rearrangements below the detection limits of conventional cytogenetics may be a common occurrence. A large series of array-CGH experiments with single HCT116 cells could clarify which of the two options is correct; however, this would be beyond the scope of this study.

It should be noted, however, that some of the differences in copy number between single-cell amplifications, at least for dividing cells, may be due to the position of each cell within the cell cycle. Cells which are harvested within S phase will have a proportion of their genome replicated and so increased in copy number when compared with cells in G_1 phase. It might be predicted that cells harvested during S phase would show higher overall variability and, therefore, noisier hybridizations which will reduce the ability to detect smaller copy number changes.

We would expect the methods described here to be equally applicable to cells microdissected from frozen sections. However, we do not yet have experience with paraffin-embedded tissue but it is likely that variability of the ratio profiles may be further increased in such histological preparations.

In conclusion, we have shown that by utilizing GenomePlex library technology for the amplification of single cells with high-resolution large insert clone array-CGH we can not only identify with confidence whole chromosome trisomies, but also detect copy number changes as small as 8.3 Mb. In this way, we demonstrate for the first time the ability of single-cell array-CGH to identify previously undetected microdeletions and microduplications and the potential for application to non-invasive prenatal diagnosis (by the analysis of fetal cells in the maternal circulation) and tumor heterogeneity.

SUPPLEMENTARY DATA

Supplementary data are available at NAR online.

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Conflict of interest statement. None declared.

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