The presence of disseminated tumor cells (DTCs) in bone marrow (BM) identifies breast cancer patients with less favorable outcome. Furthermore, molecular characterization is required to investigate the malignant potential of these cells. This study presents a single-cell array comparative genomic hybridization (SCaCGH) method providing molecular analysis of immunomorphologically detected DTCs. The resolution limit of the method was estimated using the cancer cell line SK-BR-3 on 44 and 244k arrays. The technique was further tested on 28 circulating tumor cells and four hematopoietic cells (HCs) from peripheral blood (n = 8 patients). The SCaCGH method was finally applied to 24 DTCs, three immunopositive cells morphologically classified as probable HCs from breast cancer patients and five HC controls from BM (n = 7 patients plus n = 1 healthy donor). The frequency of copy number changes of the DTCs revealed similarities with primary breast tumor samples. Three of the patients had available profiles for DTCs and the corresponding tumor tissue from primary surgery. More than two-thirds of the analyzed DTCs disclosed equivalent changes, both to each other and to the corresponding primary disease, whereas the rest of the cells showed balanced profiles. The probable HCs revealed either balanced profiles (n = 2) or changes comparable to the tumor tissue and DTCs (n = 1), indicating morphological overlap between HCs and DTCs. Similar aberration patterns were visible in DTCs collected at diagnosis and at 3 years relapse-free follow-up. SCaCGH may be a powerful tool for the molecular characterization of DTCs.

Despite many improvements in breast cancer treatment and the increase in survival time over the last decades, about 25% of the patients develop distant metastases and ultimately die of breast cancer. Consequently, there is a demand for a better understanding of the metastatic process and the identification of novel markers for tumor progression, aggressiveness and treatment susceptibility. It is still uncertain, which are the regulatory features that trigger single tumor cells to leave the primary tumor site, stay dormant, progress into metastases or to die. In parallel with additional studies of the primary tumor, an in-depth molecular analysis of tumor cells detected in the circulation (circulating tumor cells; CTCs) or in distant metastases can provide increased knowledge of the molecular basis of tumor evolution and therapy resistance.
organs (disseminated tumor cells; DTCs) would improve the ability to identify genes and signalling pathways involved in the formation of metastasis. Several studies have shown that the presence of DTCs in the bone marrow (BM) is an independent prognostic factor, and presence of DTCs at various intervals after primary diagnosis identifies patients with less favorable clinical outcome. At present, the knowledge of the heterogeneity of such cells, their interrelation and their relation to the original primary tumor is still limited (see review by Ref. 8). It is supposed that genomic profiling of DTCs may strengthen the clinical relevance of these cells and the ability to target them. This may improve treatment of minimal residual disease in the future.

Array comparative genomic hybridization (aCGH) analysis is a tool for comprehensive exploration of the tumor cell genome. However, standard aCGH is usually performed with DNA extracted from thousands of tumor cells. Consequently, adaptations of the technique for studies of individual tumor cells are required. The recently developed single-cell array comparative genomic hybridization (SCaCGH) opens for the possibility to examine copy number profiles in single-cells using high-resolution arrays. This method interferes with several steps of the SCaCGH technique, and, thus, both the ICC and the SCaCGH method require optimization before use in patient series. In addition, the low DNA quantity, the amplification step and the high resolution of matrices are expected to cause variation in the resolved copy number data, emphasizing the importance of appropriate algorithms for the genomic analysis. Accordingly, to assess reliable gains and losses correctly, there is a need for the estimation of the resolution limits of the reaction for different types of arrays.

The purpose of the study was to develop a high-resolution SCaCGH method, which can be applied for the characterization of DTCs, following currently used methods for the detection of these cells. Copy number profiles were achieved from cells of the breast cancer cell line SK-BR-3, from CTCs and from DTCs of early stage and metastatic breast cancer patients. The results indicate possibilities for new biological insight of the dissemination process.

Material and Methods
Breast cancer cell-line SK-BR-3
SK-BR-3 tumor cell-line cells were acquired from ATCC (American Type Culture Collection, Manassas, VA). The cells originate from an adenocarcinoma of the mammary gland of a Caucasian female. The cell line is hypertriploid with multiple chromosomal rearrangements.

Patient samples
Six peripheral blood (PB) samples and four BM samples were collected from 10 selected metastatic breast cancer patients included in the Micrometastasis methodology (Mm) project. The biomaterial from early breast cancer cases was collected from patients included in the Oslo Micrometastasis (MicMa) Study—Oslo1. From three patients, both BM mononuclear cells and fresh-frozen tumor tissue from the primary breast surgery were available (two from primary tumors; one from lymph node metastasis, due to lack of specimen from the primary tumor). The BM samples were collected at the time of surgery. In addition, from one of the three patients, a new BM sample, collected at 3 years relapse-free follow-up, was available. For comparative purposes, a total of 49 tumor samples from primary surgery (47 primary tumors and two lymph node metastases) were analyzed. Of these samples, 23 patients had positive DTC status at time of diagnosis, 25 patients had negative DTC status and one patient had inconclusive DTC status. Hematopoietic cells (HCs), unspecifically stained cells from negative control slides (for details see Supporting Information Methods), were provided from two patients of the Oslo Micrometastasis (MicMa) Study—Oslo1 and from one healthy donor. The studies were approved by the Regional Ethic Committee (S97103 + S00065a). All patients have signed written informed consent.

Tumor cell detection
BM was aspirated from posterior or posterior and anterior iliac crests, followed by isolation of mononuclear cells and by preparation of cytopsins for detection of DTCs by immunocytochemistry (ICC; for details, see Supporting Information Methods).

Detection protocols
NF protocol 1. Cytospins were fixed in acetone for 10 min and incubated with 80 µl of the primary antibodies AE1/AE3 or MOPC21 for 30 min, followed by 80 µl of each of the secondary and tertiary antibodies (see APAAP method in Supporting Information Methods). The antibodies were diluted in 1 × PBS buffer with 1% BSA (Sigma-Aldrich, St. Louis, MO) and cytospins were washed 2 × 5 min in TRIS-HCl buffer (0.05 M, pH 7.4, diluted 1:5 in 0.9% NaCl solution). The red color reaction on AP-bound sites was developed by transferring the slides for 10 min into a solution containing naphthol AS-Bi phosphate disodium salt (Sigma-Aldrich), tetramisole hydrochloride (Sigma-Aldrich) and the chromogen New fuchsin (NF). The reaction was followed by 30 sec of hematoxylin nuclear staining and rinsing with water. The stained slides were kept in 1 × PBS at 4°C.

BCIP/NBT protocol 2. The cytospins were incubated with blocking solution in 1 × PBS with 10% AB-serum (Bio-Rad Medical Diagnostics, Dreieich, Germany) for 20 min, followed by application of 100 µl of the primary antibodies AE1/AE3 or MOPC21 in 45 min, subsequently adding 100 µl of each of the secondary and tertiary antibodies (see APAAP method in Supporting Information Methods) with an incubation time of 30 min for each antibody. The antibodies were...
diluted in 1 × PBS with 10% AB-serum, and the cytopspins were washed 3 × 3 min in 1 × PBS buffer. The blue color reaction on AP-bound sites was developed by the application of 250 μl of a solution containing 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Bio-Rad Laboratories, Hercules, CA) and levamisole (Dako, Glostrup, Denmark), incubated for 10 min and subsequently washed in 3 × 3 min with 1 × PBS buffer and rinsed 2 × 1 min with water, as previously described.11 The stained slides were kept in 1 × PBS at 4°C. (For microdissection, polyethylene-naphthalate (PEN) membrane coated slides (Carl Zeiss MicroImaging, Jena, Germany) combined with an inverted Axiovert 40 C microscope containing 10 μl of the digestion mix. In our hands, the PEN membrane-coated slides were suboptimal for patient samples due to a tendency for cell loss and a more difficult morphological cell characterization. Isolation of cells by micromanipulation was performed on patient samples using the CellTram® Vario Microinjector and Eppendorf TransferMan NK 2 micromanipulator equipment (Eppendorf, Hamburg, Germany) combined with an inverted Axiovert 40 C microscope (Carl Zeiss MicroImaging). A video V25 C1/2° (3CCD) 0.5 × adapter together with the Axiosvision Rel. 4.8 Software (both Carl Zeiss MicroImaging) were used for photo documentation of the micromanipulation process. Because the cell size varies from patient to patient and also within the same patient, the required capillary diameter was adjusted to match the target cells. Mainly, the CustomTips Type III capillaries (Eppendorf) with a bevelled end and with an inner diameter of 25 μm were applied. Micromanipulated cells were deposited in an optical lid (Thermo Scientific, Waltham, MA) containing 4.9 μl nuclease free water, closed with an Eppendorf PCR tube (Eppendorf), centrifuged for 10 sec at 6,708 g and stored at −20°C. Several slides were tested for micromanipulation: adhesion slides (Thermo Scientific, Hamburg, Germany), poly-L-lysine (Sigma-Aldrich)-coated SuperFrost slides (Glaswarenfabrik Karl Hecht, Sondheim, Germany) and SuperFrost plus slides (Thermo Scientific). In principle, all types of slides could be used for the cell isolation and in the downstream amplification reaction. However, a selective loss of tumor cells observed for adhesive slides and the labor of manual coating of poly-L-lysine slides convinced us to prefer the SuperFrost plus slides for micromanipulation experiments. To prevent stickiness of the capillaries used for micromanipulation, coating with different solutions such as Repel-Silane ES (GE Healthcare, Chalfont St Giles, UK), Sigmacote® (Sigma-Aldrich) and BSA (Roche Diagnostics, Mannheim, Germany) was examined. It turned out that for CTCs coating with BSA was the preferable agent to prevent blockage, whereas DTC isolation usually worked satisfactorily without any coating.

Amplification

All samples were amplified using the GenomePlex® Single-Cell Whole Genome Amplification Kit (Sigma-Aldrich). Single-cell lysis and fragmentation, as well as the library preparation, were performed according to the producer’s instructions. For the amplification, the following mix was added: 7.5 μl of 10 × amplification master mix, 51 μl nuclease free water and 1.5 μl Titanium Taq DNA polymerase (1 μl of enzyme per 50 μl reaction equals to 15–25 U, BD Biosciences Clontech, Heidelberg, Germany). In a thermal MJ Mini cycler (Bio-Rad Laboratories), the samples were first denatured at 95°C for 3 min, followed by 25 cycles, each consisting of a denaturation step at 94°C for 30 sec and an annealing/extension step at 65°C for 5 min. Amplified samples were stored at 4°C. According to the whole genome amplification protocol, a positive control DNA (Control Human Genomic DNA) from the amplification kit (Sigma-Aldrich) and two negative control samples (Aqua bidest) were performed alongside with each experiment. Clean up was performed with the Gen elute™ PCR clean up kit (Sigma-Aldrich) according to the producer’s instructions. DNA concentrations were measured using the NanoDrop® ND-1000 (Thermo Fisher Scientific, DE). Neither a reduction of the annealing temperature nor an increased amount of Titanium Taq DNA polymerase significantly increased the DNA yield substantially (data not shown).

Array comparative genomic hybridization

From the tumor tissue samples collected at primary surgery, DNA was extracted using an ABI 341 Nucleic Acid Purification System (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. For the analysis of the SK-BR-3 cells, the ScaCGH method was performed as published previously.10,11 DNA from the samples was analyzed using either Agilent’s Human Genome CGH Microarrays 44k, 244k or both (Agilent Technologies, Santa Clara, CA).16 Experiments were performed with either female or male (for DTCs from metastatic patients) human genomic reference DNA (both Human Genomic DNA, Promega, Madison, WI) following the standard protocol. For scanning of the signals, the Agilent DNA Microarray Scanner with Surescan High-Resolution Technology was applied. Scanned microarray images were read and analyzed with Feature Extraction v9.5 or v10.7.3.1
(Agilent Technologies), using different protocols (CGH-v4_95_Feb07, CGH-v4_10_Apr08 and CGH_105_Dec08) for aCGH-preprocessing, which included linear normalization. Data are stored in the GEO database (accession number GSE27574). We compared the results of different DNA concentrations for the array reaction (60, 120 and 180 ng/μl on a NanoDrop 3300 Fluorospectometer, Thermo Scientific) and the necessary DNA concentration after single-cell amplification was found to be at least 180 ng/μl.

**Statistical analysis**

The identification of chromosomal gains and losses was based on fitting piecewise constant curves to the data using a penalized least squares criterion, with the use of the Piecewise Constant Fit (PCF) algorithm, also known as a Potts filter. Each chromosomal arm was separated into a set of distinct segments, defined by a minimum of 25 probes. Within each segment, the height of the curve was defined by the mean of the probe values. To identify gains and losses for frequency plots, a threshold to the curves defined by the PCFs was applied. For most single-cells from the patient samples, a fraction of the probe values had very low values. To avoid a too strong influence from these probes in the copy number estimation, a lowest detectable value was defined, and all values below this limit were set equal to the limit (typically \( \frac{C_0}{2} \)). Following this procedure, the zero line was redefined to give an average value of 0 across all chromosomes for each cell. It was furthermore observed that data from single DTCs showed systematic fluctuations in probe values, probably due to low amplitude and short or moderate length (similar relations are seen in other chromosomes). Two examples of small copy number gains found in all plots are indicated by green arrows in the large amount of cells plot. The resolution limit depends both on the length of the aberration and on the signal-to-noise ratio. The arrows mark aberration intervals of height 1–1.5 times the SD of the single-cells and with length 0.8–1.3 Mb; these are at the limit of what was detectable (to be reliably identified an aberration of height 1 SD should be 2–3 Mb).

Figure 1. Genomic profiles of single-cells, a pool of single-cells and a large amount of cells of the cancer cell line SK-BR-3. Estimated copy numbers for chromosome 8 for SK-BR-3 cells using 244k arrays are illustrated. Data for three single-cells were compared to a pool of collected single-cells (20–30 cells) and a large amount of cells, respectively. Note that the major aberrations detected in the cell pool are seen also in the single-cells, while some details of the aberration pattern are lost, mainly due to low amplitude and short or moderate length (similar relations are seen in other chromosomes). Two examples of small copy number gains found in all plots are indicated by green arrows in the large amount of cells plot. The resolution limit depends both on the length of the aberration and on the signal-to-noise ratio. The arrows mark aberration intervals of height 1–1.5 times the SD of the single-cells and with length 0.8–1.3 Mb; these are at the limit of what was detectable (to be reliably identified an aberration of height 1 SD should be 2–3 Mb).

Figure 2. Illustration of the ScaCGH method. The different steps involved in the ScaCGH process are shown: sample preparation of CTCs and DTCs, micromanipulation (see also Supporting Information Fig. 3), single-cell whole genome amplification, aCGH performance and aCGH analysis.
to the amplification process. These fluctuations were similar across all cells, including the HCs. To avoid false aberrations due to these fluctuations, an initial step in the estimation was to construct a moving average based on the available HCs. For each probe value from the DTCs, the corresponding moving average value was subtracted. To perform hierarchical clustering of single-cells, an extension of the PCF method (multiPCF) was used, in which the least square fit is found simultaneously on all cells and with the same breakpoints across cells. The copy number estimates (the mean values) on the segments between these common breakpoints were then the basis for the clustering. The clustering itself was based on the Ward method with standardized variables. The multiPCF method was further used in the examinations of the similarities between single-cells and the tissue from the primary tumor surgery, because one may then compare mean values on equally delineated segments.

The entire genomic profile of each individual sample was analyzed separately, and chromosomes presented in the figures were selected on the basis of the aberrations detected in primary disease and/or typically involved in genomic changes in breast cancer.

**Figure 3.** Frequency plot of copy number changes in primary tumors and occult tumor cells. The frequency of copy number changes using 244k arrays and a threshold of 0.4 are illustrated for the chromosomes 1–22 of (a) 49 tissue samples from primary surgery (47 from primary tumor, two from lymph node metastasis) from early stage breast cancer patients and of (b) 24 DTCs plus three immunopositive cells morphologically classified as probable HCs (from three early stage breast cancer patients (the primary tumor tissue from these patients are included in a) and from four metastatic breast cancer patients). Amplified regions are marked in red, and deleted regions are marked in green. The major high frequency peaks are visible including gains at 1q, 8q and 17q (indicated by red arrows) as well as losses at 8p and 11q (indicated by green arrows), respectively. Several additional aberrations in the DTCs were also detected, and some of these may be due to noise or the moderate number of patients. For the segmentation the PCF algorithm was applied. In the calculations of frequency, DTCs are weighted, so that each patient contributes equally to the frequency plot to balance differences in numbers (using all DTCs unweighted revealed similar structures. The X-chromosome was excluded, because male aCGH references were used on a subset of the samples).

<table>
<thead>
<tr>
<th></th>
<th>Total number of DTCs</th>
<th>Number of DTCs with profile similar to primary disease</th>
<th>Number of DTCs with balanced profiles</th>
</tr>
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<tbody>
<tr>
<td>MicMa003</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>MicMa083</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>MicMa107</td>
<td>$10^1$</td>
<td>$7^1$</td>
<td>$3^1$</td>
</tr>
</tbody>
</table>

1DTCs and probable HCs.

**Florescence in situ hybridization**

Florescence in situ hybridization (FISH) analysis was performed on SuperFrost plus slides containing BM mononuclear cells from patients with early stage of the disease (MicMa003, MicMa083 and MicMa107; for details, see Supporting Information Methods).

**Results**

**Pilot studies using cells of the breast cancer cell line SK-BR-3**

Before the analysis of the genomic profile of DTCs from patient samples, we estimated the robustness and resolution...
limits of the SCaCGH method using the breast cancer cell line SK-BR-3. To determine resolution differences and restrictions, we applied DNA from (1) single-cells, (2) a pool consisting of 20–30 single-cells and (3) a large amount of cells, to 44k and 244k arrays. The obtained profile from the large number of cells was supposed to show the correct copy number variation (CNV) profile of the SK-BR-3 cell line.

Figure 4.
number changes, and the estimates from the single-cells were compared to this profile (Supporting Information Table 1). The major copy number gains and losses were detected in the single-cells (Fig. 1); however, some fine structures may be lost due to the higher noise level of the single-cells (Supporting Information Fig. 1).

The resolution limit for the 244k arrays was estimated by comparing the aberrations detected in the single-cells to those found in a large amount of cells, supplemented by simulation studies using artificial aberrations added to the real tracks. The resolution for an aberration of magnitude equal to 1 standard deviation (SD) was in the order of 2–3 Mb using 244k arrays. Figure 1 shows examples of such detected short aberrations as well as some even shorter aberrations or aberrations with lower magnitude that remained undetected. Comparing the results for 44 and 244k arrays, the SD of the probe values were roughly 20% higher for the 244k than for the 44k data. However, given that the probe positions are about five times denser for 244k than for 44k arrays, 244k gives clearly higher resolution.

Adjustments of the SCaCGH method
The principles for the SCaCGH method are presented in Figure 2. Various steps of the initially used SCaCGH method required adjustments for genomic profiling of DTCs. Initial testing and method adaptation were conducted using the cancer cell line SK-BR-3, followed by 28 CTCs and four HCs from six metastatic and two early breast cancer patients (Supporting Information Table 2 and Fig. 5).

Micromanipulation, amplification and aCGH could be reproduced at different locations (for details, see Fig. 2; and Supporting Information Tables 2 and 3). The SCaCGH method may be affected by staining and fixation steps, possibly disturbing downstream applications. Therefore, two different staining protocols (NF protocol 1 and BCIP/NBT protocol 2) were examined using SK-BR-3 cells. The highest DNA concentrations were obtained for methods implementing BCIP/NBT as chromogen (199.2 and 211.0 ng/µl, respectively). The protocols with NF, with or without acetone and/or hematoxyline, resulted in lower DNA quantities (44.6–83.4 ng/µl, see Supporting Information Fig. 4). The results revealed that BCIP/NBT did not interfere with the amplification process, and thus the BCIP/NBT protocol 2, including omission of hematoxylin, was chosen for subsequent SCaCGH analysis. However, to some extent, this protocol resulted in a slightly higher number of immunopositive cells and a more difficult morphological classification of the cells as DTCs or false-positive HCs (data not shown).

SCaCGH analysis of DTCs from breast cancer patients
Altogether, the SK-BR-3 results, the adaptations and the initial testing of CTCs revealed the ability to isolate one single tumor cell from a background of HCs, amplify the genomic material and detect aberrations by aCGH. Consequently, the method was used on DTCs from breast cancer patients. To compare resolution differences between 44k and 244k arrays, seven samples were analyzed on both 44k and 244k arrays (Supporting Information Table 3). Similar aberration patterns were detected (Supporting Information Fig. 2). However, the 244k array is still considered to have potential for the detection of smaller aberrations, and, consequently, this platform was selected for the following investigations. The resolution for the DTCs was lower than the limit found for SK-BR-3 cells, but still clearly high enough for the comparison of DTCs to each other and to the primary disease. This was also facilitated by the analysis of several DTCs from the same patient; thus, comparison could be performed using multi-sample methods (the multiPCF method).

The adjusted SCaCGH method was used to analyze the copy number profiles of 24 DTCs, three immunopositive cells morphologically classified as probable HCs and five HCs controls from seven breast cancer patients and one healthy donor using 244k arrays (Supporting Information Table 3). Genomic aberrations of the 24 DTCs and three probable HCs exhibited gains and losses similar to the major changes detected in the primary breast tumors (gains at 1q, 8q and 17q as well as losses at 8p and 11q, respectively). Several additional high-frequency aberrations among the DTCs were also found (Fig. 3).

Figure 4. Genomic profiles of DTCs and the corresponding primary disease. Copy number profiles obtained by SCaCGH using 244k arrays, from a patient with early stage breast cancer are shown for (a) tumor tissue from primary surgery (in this patient lymph node metastasis), (b) six immunostained cells from samples collected at time of diagnosis and (c) four immunostained cells from a sample collected at 3 years relapse-free follow up. Pictures of the examined, immunostained DTCs are placed next to their copy number profiles. According to the immunomorphological evaluation were in (b) four cells categorized as DTCs and two cells as probable HCs and in (c) three cells categorized as DTCs and one cell as probable HC. The majority of these candidate immunostained cells (7 of 10) showed clear similarity to the genomic changes and aberration breakpoints found in the primary disease profile, only three cells had a balanced copy number profile. The copy number gains detected in the tumor tissue from primary surgery and DTCs at chromosome 8 and 17 were further supported by FISH analysis: (d) FISH results of one immunomorphologically classified DTC (left picture) showed four green signals for the probe GPR172A (right picture, indicated by horizontal green arrows), located at 8q24.3, 165.49 Mb. (The cell was hybridized in parallel with another FISH probe for SNF8, located at 17q21.32 resulting in five signals, these results are illustrated in Supporting Information Fig. 9). Different single-, multichannel- and DAPI-images of the cell are shown in Supporting Information Figure 10. Weak spots are most likely minor binding sites caused by cross-hybridization. FISH signals were also seen in many of the present normal hematopoietic bone marrow cells and in immunostained cells with HC morphology (false positive cells). These cells showed two signals of each probe, compatible with normal, nonamplified regions.
Examination of DTCs from three early breast cancer patients in relation to each other and to the corresponding tumor tissue from primary surgery revealed that major copy number gains and losses detected in the tumor tissue could be recovered in 12 of 18 DTCs and probable HCs (11 of 15 DTCs) (Table 1 and Figs. 4 and 5; Supporting Information Figs. 7–9).

Cluster analysis of the DTCs showed that most cells of the same patients were more related to each other than to DTCs from other patients (Supporting Information Fig. 6). Interestingly, similarities in the aberration profiles, to each other and to the tumor tissue, were found in the DTCs from time of diagnosis and in those collected at 3 years relapse-free follow-up (Table 1, Figs. 4 and 5; Supporting Information Fig. 9). However, minor differences between DTCs and the primary tumor could also be indicated (Supporting Information Fig. 7). Three immunostained cells of patient MicMa107 were in their primary morphological characterization classified as being probable HCs. Two of these cells showed balanced profiles, similar to HCs and one cell revealed copy number changes similar to the tumor tissue and DTCs (Fig. 4 and Supporting Information Fig. 9).

To further support the identified genomic regions of copy number changes, FISH analysis on DTCs was performed, using probes from selected regions based on the aCGH results. Regions of copy number gains could be detected by FISH using additional DTCs of the same patients (Fig. 4; Supporting Information Figs. 9 and 10).

**Discussion**

Here, we present a suitable method for the genomic characterization of single DTCs and CTCs from patient samples. To confirm the utility of the technique, a total of 24 DTCs, three probable HCs and nine HCs from breast cancer patients or healthy donors were analyzed. The majority of DTCs with aberrant profiles showed genomic changes with high concordance to other tumor cells of the same patient and, for those so far available, to the corresponding tumor tissue from primary surgery, while a fraction of DTCs revealed balanced copy number profiles. A frequency plot of gains and losses determined in DTCs showed patterns as typically seen in primary breast tumors.

The use of high-resolution arrays, as presented in the current study, provides the opportunity to explore the level of genomic aberration overlap and dissimilarities between DTCs and the primary tumor. Such an approach may provide important information about the biology behind the dissemination process. Two principally different models have been proposed for the dissemination of tumor cells from the primary tumor: a linear progression model considering cell dissemination as a rather late event in tumorigenesis in contrast to a parallel progression model proposing tumor cell dissemination as an early event (see review by Ref. 21). The latter hypothesis are supported by the observation of some authors that the genomic aberrations are different in the primary tumor and in DTCs. Using classical CGH and a cytokeratin (CK2 and A45-B/B3) immunostaining protocol, one of these studies found in breast cancer that just two of 14 matched pairs of primary tumors and DTCs showed a high degree of similarity. Interestingly, samples from the regional lymph node metastases in this study closer related to the primary tumors than to DTCs, and thus the authors conclude that tumor cells may disseminate in a less progressed state, acquiring copy number changes at a later stage. Previous studies have used low-resolution classical CGH (see review by Ref. 8), while the results of the high-resolution arrays presented in our study indicate large degree of similarities between DTCs and the tumor tissue from primary surgery, having in mind that one of the samples were from an axillary lymph node metastasis. Consequently, our results may support the linear progression model, but the possibility for early DTC dissemination cannot be excluded. The pattern of dissemination/tumor progression might also vary in different subtypes of breast cancer. As part of the original study design, an additional BM sample collected 3 years after the diagnosis was available for one of the patients. Interestingly, the aberration patterns of the examined DTCs at 3 years of relapse-free follow-up showed exactly the same changes as detected in the primary disease. This indicates that the “dominant” cells from the primary disease were able to stay dormant for several years. It has been shown that tumor cells can obtain aberrations of potential clinical relevance during the metastatic process. Although the present results show high degree of overlap between DTCs and the primary tumors, differences were also indicated. For further biological insight, thorough testing in larger patient series including a larger number of cells is needed to disclose the level of heterogeneity between single tumor cells from a patient and between these cells and the primary tumor. Detailed studies of primary tumor heterogeneity are also required.

The SCA CGH process implies several methodological challenges, including a low-initial amount of DNA, an obligatory amplification step and high-resolution arrays, resulting in an increased level of noise, issues also raised by other authors. Data from our single-cell experiments showed systematic fluctuations in probe values, probably originating from the amplification process. These fluctuations were similar across cells, including the HCs. A moving average based on the available HCs was constructed to avoid false aberrations due to the fluctuations. Then, for each probe value from the DTC, the corresponding moving average value was subtracted. For initial testing and method adaptation, SK-BR-3 and CTCs were applied. Our previously published data suggest that high-resolution oligo-arrays allow a reliable identification of aberrations as small as ~3.0 Mb in single-cells. A similar or slightly higher resolution was seen for the SK-BR-3 cells. The resolution for DTCs is currently lower, but high enough for the comparisons of larger scale aberration patterns. Aberration detection in the DTCs was facilitated by the available copy number information of the primary disease. Combined analysis of profiles of DTCs and the primary tumors may enhance
the possibility to explore novel changes generated during the dissemination process. A step to increase the resolution and even to allow the detection of allelic imbalances would imply next-generation sequencing, as proposed by others. However, in addition to high expenses, this extension would also require further technical adaptations and methodological refinements.

Figure 5. Copy number profiles of DTCs collected at 3 years of relapse-free follow up. Copy number profiles are shown for the tumor tissue from primary surgery and four DTCs collected at 3 years relapse-free follow-up of a patient with early stage breast cancer. SCaCGH data using 244k arrays are shown estimating copy numbers for chromosome 13. The analysis were carried out using the multiPCF algorithm, implying that the breakpoints are equal for the tumor tissue and the single-cells, while the height of the curves corresponds to the mean value of the probes within each segment. Note that the aberrations present in the tumor tissue are recovered in DTCs with great similarity.
Furthermore, methodological challenges are the potential for DNA degradation reducing the amplification success. This problem can be minimized by performing the DNA amplification relatively shortly after the isolation of the single tumor cells. In addition, the stickiness of the neighboring cells (if present) may affect the isolation of one single intact tumor cell. This can be prevented by decreasing the total number of cells per slide. Adaptation of the SCAcGH method for DTCs is particularly challenging due to the need for an ICC detection step before the isolation of the single-cell. This process can interfere with the DNA amplification (Supporting Information Fig. 4). However, a careful testing of various fixation and staining options revealed an ICC protocol resulting in satisfactory yield of amplified DNA.

Several cells immunocytochemically and morphologically classified as DTCs had balanced patterns, as also shown by others using classical CGH.23 Probably, some or most of the latter cells are false-positive HCs, due to the morphological overlap between tumor cells and HCs.30,31 The required adjustments of the ICC method, using the chromogen solution BCIP/NBT instead of the commonly used NF and omission of nuclear counterstaining, probably lowered the specificity of the ICC staining and thus increased the number of false-positive cells. Our experience was that BCIP/NBT did not reproduce cellular details to the same degree as NF. Moreover, we determined the copy number profiles of three selected immunostained cells, which had been scored as probable HCs by immunomorphological evaluation. Interestingly, one of these cells showed copy number changes similar to the tumor tissue and DTCs, while the other two cells revealed balanced profiles similar to HCs. Correct identification of DTCs based on immunomorphology is therefore challenging and further characterization is important.

In conclusion, the presented SCAcGH method is a promising tool for the recognition of the genetic signatures of occult tumor cells. This may open for enhanced understanding of the biology of tumor dissemination and may be of value for future improvements in breast cancer treatment. In addition, genomic analysis of DTCs will improve the specificity of DTC detection, which is of utmost importance for minimal disease research.

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