Isolation and Characterization of Circulating Tumor Cells Using a Novel Workflow Combining the CellSearch® System and the CellCelector™

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Circulating tumor cells (CTC) are rare cells which have left the primary tumor to enter the blood stream. Although only a small CTC subgroup is capable of extravasating, the presence of CTCs is associated with an increased risk of metastasis and a shorter overall survival. Understanding the heterogeneous CTC biology will optimize treatment decisions and will thereby improve patient outcome. For this, robust workflows for detection and isolation of CTCs are urgently required. Here, we present a workflow to characterize CTCs by combining the advantages of both the CellSearch® and the CellCelector™ micromanipulation system. CTCs were isolated from CellSearch® cartridges using the CellCelector™ system and were deposited into PCR tubes for subsequent molecular analysis (whole genome amplification (WGA) and massive parallel multigene sequencing). By a CellCelector™ screen we reidentified

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97% of CellSearch® SKBR-3 cells. Furthermore, we isolated 97% of CellSearch®-proven patient CTCs using the CellCelector™ system. Therein, we found an almost perfect correlation of $R^2 = 0.98$ (Spearman's rho correlation, $n = 20$, $p < 0.00001$) between the CellSearch® CTC count ($n = 271$) and the CellCelector™ detected CTCs ($n = 252$). Isolated CTCs were analyzed by WGA and massive parallel multigene sequencing. In total, single nucleotide polymorphisms (SNPs) could be detected in 50 genes in seven CTCs, 12 MCF-7, and 3 T47D cells, respectively. Taken together, CTC quantification via the CellCelector™ system ensures a comprehensive detection of CTCs preidentified by the CellSearch® system. Moreover, the isolation of CTCs after CellSearch® using the CellCelector™ system guarantees for CTC enrichment without any contaminants enabling subsequent high throughput genomic analyses on single cell level. *Biotechnol. Prog.*, 33:125–132, 2017
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Introduction

Already in 1869, Thomas Ashworth described "a case of cancer in which cells similar to those in the tumors were seen in the blood after death" and proposed the presence of circulating tumor cells (CTCs) as prerequisite of metastasis.[1] However, many decades passed before suitable detection and isolation methods have facilitated the characterization of CTCs. Isolation and characterization of CTCs is of great importance to improve cancer diagnosis and prognosis as well as to step toward a more personalized cancer therapy. CTCs possess the capacity to form metastases[2] and their quantitative appearance in the blood correlates with reduced progression-free and overall survival.[3] Moreover, quantification of CTCs in breast cancer is of higher prognostic value than conventional imaging[4] and serves as *liquid biopsy* enabling real-time monitoring of therapy success.[5] Therein, CTCs might rather resemble secondary lesion characteristics than the primary tumor and give information about tumor progression in a fast and less invasive fashion compared to sequential biopsies.

Because of the estimated frequency of 1 CTC in 10^6-10^8 white blood cells, detection and quantification of CTCs is very challenging. For epithelial cells expressing the epithelial surface protein EpCAM isolation could be standardized with the CellSearch® device (Janssen Diagnostics LLC, Raritan, NJ). This system uses ferrofluid coupled to EpCAM-specific antibodies to capture fixated CTCs which are then identified by immunofluorescent detection of cytokeratin expression. Although its application in CTC detection is undisputable, isolation and subsequent downstream analyses of single CTCs after CellSearch® is still challenging: CTCs are trapped in a cartridge with a massive background of cocaptured leukocytes (approximately 1×10^4/cartridge). The big hurdle for further downstream analysis of CTCs has been to isolate them with high efficiency from these cartridges. This demand has been recognized by the industry and several techniques have been developed such as fluorescence activated cell sorting (FACS),[6] DEPArray[7] or different micromanipulation systems. Here we present a robust workflow combining capturing and immunostaining of CTCs (CellSearch®) and their isolation with automated micromanipulation (CellCelector™). Successful downstream whole genome amplification (WGA) and massive parallel next generation sequencing proved high quality of isolated CTCs.

Material and Methods

Cell lines and culture conditions

MCF-7 and T47D breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were cultured in RPMI 1640 containing 10% fetal calf serum and 1% Penicillin-Streptomycin (all Gibco, Karlsruhe, Germany). Culture medium for MCF-7 was supplemented with 25 mM HEPES (Gibco); for T47D cells 10 mM HEPES, 1 mM sodium pyruvate (Gibco), and 0.45% D-(+)Glucose solution (Sigma-Aldrich, Munich, Germany) was added. All cells were grown at 37°C in a humidified atmosphere with 5% CO2. Additionally, prestained and fixed SKBR-3 breast carcinoma cells from the CellSearch® Epithelial Cell Control Kit (Janssen Diagnostics, LLC, USA) were used for validation of single cell isolation experiments.

Patient material

Blood samples were collected within the German DETECT III study (NCT01619111) from patients with primarily HER2-negative metastatic breast cancer. Written informed consent was obtained from all participating patients and the study was approved by the local ethics committees (525/2011AMG1). Blood withdrawal was performed into CellSave Preservative tubes and samples were processed within 72 h.

Enrichment and quantification of CTCs

For CTC enumeration, the FDA-approved CellSearch® system was used. This system allows automated enrichment and immunostaining of CTCs. Whole blood (7.5 mL) was processed using the CellSearch® Circulating Tumor Cell Kit (Janssen Diagnostics). In brief, immunomagnetic enrichment of fixed cells was achieved via an anti-EpCAM ferrofluid. Enriched cells were stained with the nucleic acid dye DAPI and monoclonal antibodies specific for the epithelial marker cytokeratin (CK, anti-CK/PE) and the leukocyte marker CD45 (anti-CD45/APC). After staining, captured cells were transferred into a cartridge within a MAGNEST® magnetic holder and were microscopically scanned in the CellTracks Analyzer II® (CK-PE: 200 ms, DAPI: 30 ms, CD45-APC: 600 ms). Subsequently, CTCs were identified by trained and certified operators via DAPI and cytokeratin positivity, and CD45 negativity.[8] All samples were stored at 4°C in the dark and were processed within 1 week.

Isolation of CTCs/single cells using the CellCelector™

The CellCelector™ (ALS GmbH, Jena) comprises an inverted microscope (CKX41, Olympus) with a CCD camera system (XM10-IR, Olympus), a robotic arm for single cell

micromanipulation including a glass capillary, and an automated stage for the mounting of different culture dishes.[9] After CellSearch® CTC enrichment, the cartridge contents were pipetted onto glass slides by using gel loading tips (MμltiFlex®-Tips 1–200 μL; Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Once the cells have settled to the bottom, microscopic analysis was performed using the CellCelector™ software 3.0 (ALS, Jena, Germany).

Imaging. The samples were scanned with a cross-stage speed of 20% and 20× magnification in the following illumination channels: DAPI (for visualization of nuclei), TRITC (for CK), and Cy5 (for CD45). The following exposure times were used: 50 ms for DAPI, 300 ms for TRITC, and 500 ms for Cy5. For staining of cell lines an anti-CK antibody labeled with FITC (pan-Cytokeratin-FITC [C11], Genetex, Irvine, CA, USA) instead of PE (CellSearch®) was used. Incubation time was 1 h at RT.

For cell isolation, the images of single cells scanned in all fluorescence channels (BF, DAPI, FITC, TRITC, Cy5, all in 40× magnification, same exposure times as stated above) were stored for later documentation.

Selection criteria. The scan result with the list of positive target cells was analyzed using the two scan parameters *diameter* (signals ranging from 4 to 40 μm) and *gray value mean* (fluorescence intensity of >2,000 for CK signal). Only DAPI/CK-positive and CD45-negative events were included, all others were disabled from the result list. Moreover, events were filtered according to their morphological criteria in 40× magnification in the bright field (BF) and fluorescence (FL) illumination channels. In BF, a CTC event should occur as a round-shaped cell. Besides, signals in the different fluorescence channels (DAPI, FITC/TRITC) should not overlay one another completely, whereas the DAPI signal should be located more or less inside the CK signal without any sign for DNA fragmentation which is indicating apoptosis. Candidate cells for picking have been selected by having no neighboring cell in a radius of 75 μm visible within the search window. The search window is given by the software displaying an area of 1 mm^2. Therein, the optimal cell density for tumor cell picking—devoid of contaminating leukocytes—is approximately 15 cells/1 mm^2.

Cell isolation parameters. Imaging in the DAPI channel (40× magnification) was used for cell isolation ("picking") to control the picking process and to ensure that the target was retrieved. After positioning the glass capillary 15–25 μm above the glass surface, the cells were aspirated with a volume of 20–100 nL using a 30 μm glass capillary. For optimal cell deposition, PBS buffer (2–9 μL) was taken up into the capillary prior to the picking process. Aspirated cells were deposited into PCR tubes which were prefilled with 50–100 μL PBS buffer. The complete cell isolation process (sample loading, scanning, analyzing, isolation, and deposition) for 50 CTCs took approximately 1–2.5 h guaranteeing fast processing of samples. Finally, the PCR tubes containing single cells were centrifuged for 10 min at 1300 rpm and the supernatant was removed leaving 1 μL of PBS in the tube. The cells were stored at −80°C until further use.

Whole Genome Amplification (WGA)

Whole genomes of isolated single cells were amplified using the Ampli1™ WGA Kit according to the manufacturer's protocol (Silicon Biosystems, Bologna, Italy). Herein, the 1 day protocol with an extended overnight step for cell lysis was performed. For quality control, 1 μL of WGA products were analyzed utilizing the Ampli1™ QC Kit which assays the integrity of four genomic loci in a multiplex PCR. As positive control, genomic DNA from cell lines was used. QC-PCR products were separated and visualized in a 1.5% agarose gel. Whole genome amplified samples displaying all four specific bands were used for sequencing.

Sequencing

Library preparation and semiconductor sequencing. For library preparation, the multiplex PCR-based Ion Torrent AmpliSeq™ technology (Life Technologies) with the Ampli1™ CHPCustom Beta panel (kindly provided by Silicon Biosystems) was used. This panel was designed to target 315 amplicons covering mutations from 50 tumor suppressor genes and oncogenes (e.g., *AKT1*, *ALK*, *APC*, *AR*, *ATM*, *BRAF*, *CDH1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FGFR1*, *FGFR2*, *FGFR3*, *IDH2*, *JAK3*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *MYC*, *NOTCH1*, *NRAS*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *SMAD4*, *TP53*, *VHL*).

Amplicon library preparation was performed with the Ion AmpliSeq™ Library Kit v2.0 using approximately 10 ng of DNA. Briefly, the DNA was mixed with the primer pool for the 315 amplicons and the AmpliSeq™ HiFi Master Mix prior to PCR with a BioRad PCR cycler (Munich, Germany). Subsequently, primer end sequences were partially digested using FuPa reagent, followed by the ligation of barcoded sequencing adapters (Ion Xpress Barcode Adapters, Life Technologies). The final library was purified using AMPure® XP magnetic beads (Beckman Coulter, Krefeld, Germany) and quantified using qPCR (Ion Library Quantitation Kit, Thermo Fisher Scientific, Waltham, USA) on a StepOne qPCR machine (Thermo Fisher Scientific). The individual libraries were diluted to a final concentration of 100 pM and processed to library amplification on Ion Spheres using Ion PGM™ Template OT2 200 Kit (Thermo Fisher Scientific). Unenriched libraries were quality-controlled using Ion Sphere quality control measurement on a QuBit instrument (Thermo Fisher Scientific). After library enrichment (Ion OneTouch ES), the library was processed for sequencing using the Ion Torrent 200 bp sequencing v2 chemistry and the barcoded libraries were loaded onto a chip. Our way of pooling eight samples on a 318v2 chip resulted in a mean coverage of 3000-fold per amplicon.

Variant calling and annotation. Data analysis was performed using the Ion Torrent Suite Software (version 4.4). After base calling, the reads were aligned against the human genome (hg19) using the TMAP algorithm within the Torrent Suite. Variant calling was performed with the variant caller plugin within the Torrent Suite Software and the IonReporter package using a corresponding bed-file containing the coordinates of the amplified regions. Only variants with an allele frequency >5% and minimum coverage >100 reads were taken into account. Variant annotation was performed using Annovar.[10] Annotations included information about nucleotide and amino acid changes of RefSeq annotated genes, COSMIC and dbSNP entries as well as detection of possible splice site mutations. For data interpretation and verification, the aligned reads were visualized using the IGV browser (Broad Institute).[11]

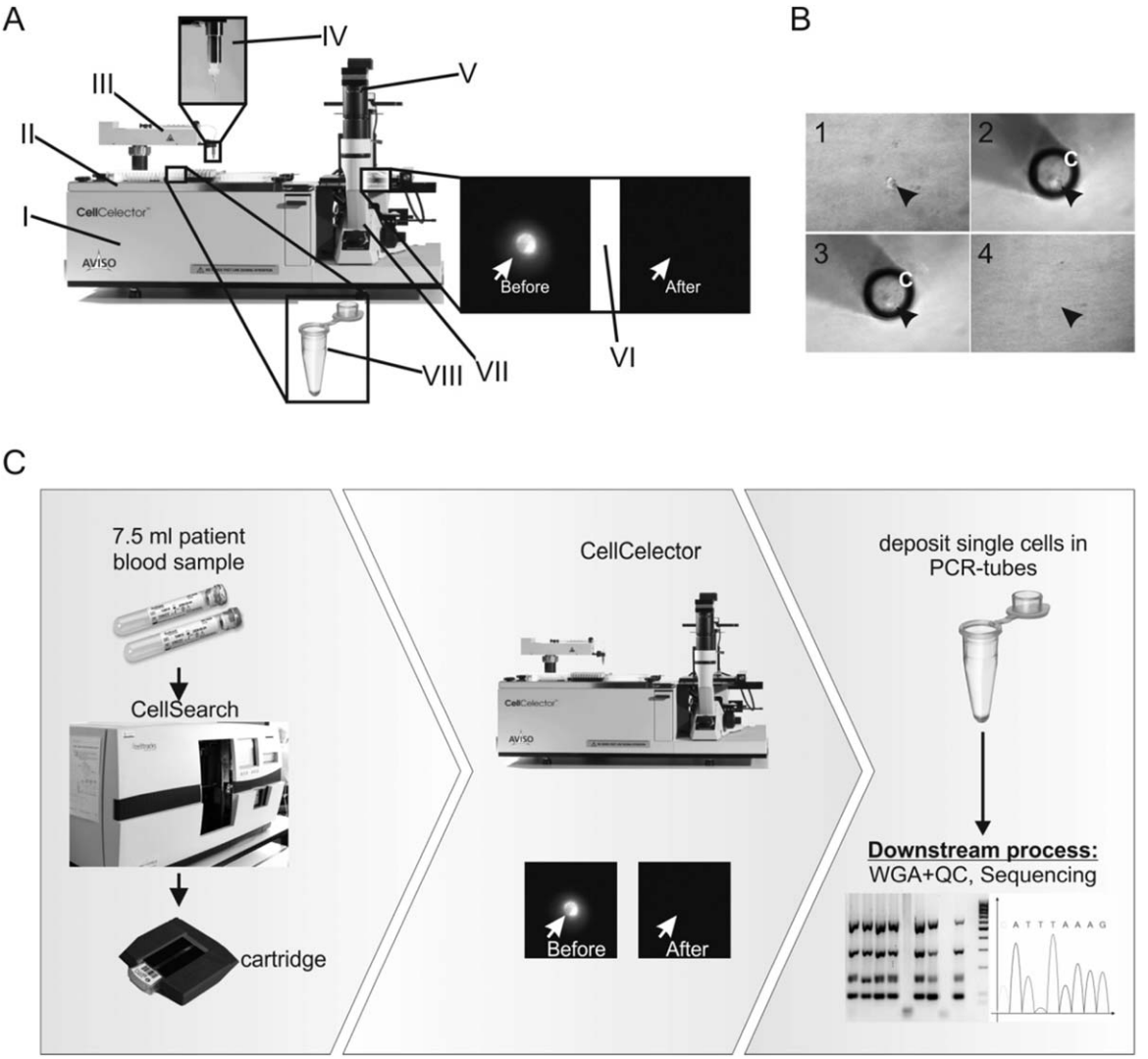

Figure 1. Experimental workflow for the detection, isolation and molecular characterization of CTCs combining the CellSearch® system and the CellCelector™ device.

(A) The CellCelector™ is basically composed of two elements: an automated micromanipulation/robotic device (I–III) and the optical component (V and VII). The basic unit (I) provides the so-called decktray (II) where the deposition platforms and reservoirs for buffer/ sterilization solutions are located (II). Deposition positions are 96-well plates, glass slides or PCR tubes (VIII). The device arm (III) possesses a 25 µL Hamilton syringe and holds a glass capillary (20–50 µm in diameter) (IV). The syringe is connected via a tubing system to the capillary. The optical system is composed of an inverted microscope (Olympus-CKX41) (VII) and a monochromatic CCD-camera (V). The cross-stage is located above the objectives enabling the visual control of the cell isolation (VI). In VI a cell isolation in bright field is shown, the horizontal lines surrounding the cell represents the ferrofluid, which aligns due to the applied magnetic field. (B) Short cuts of CTC isolation. A CTC (arrow head) is focused by the software (1) and the capillary (c) moves with constant and gently speed over the cell (2), aspirates with 25 nL (3) and isolates the cell (4). (C) Workflow to isolate EpCAM-positive CTCs after CellSearch® using the CellCelector™. Blood samples (7.5 mL) are processed by the CellSearch® system which results in the enrichment of EpCAM-positive CTCs within a cartridge. CellSearch® sample is scanned. After scanning the CellSearch® sample by the optical system of the CellCelector™, particles fulfilling CTC criteria are isolated and transferred into PCR tubes until further use (whole genome amplification and sequencing).

Statistics

Regression analysis and heat map generation were performed using the statistical computing language and environment R.[12] To minimize false positive events, single nucleotide polymorphisms (SNPs) were required to have sequencing depth of at least 100×, an allele frequency of at least 5% and a quality score (provided by the software) of >1,000. We considered only variants with the above mentioned criteria for heat map generation.

Results and Discussion

CellSearch®-CellCelector™ workflow

Single cell/CTC isolation is still very challenging, since most of the CTC enumeration methods merely allow for CTC enrichment, but not for CTC isolation. Moreover, CTCs are rare events in the background of supernumerary blood cell components and constitute a very heterogeneous population differing in their protein expression, morphology and size. Therefore, comprehensive CTC characterization requires optimized workflows, starting from enrichment and isolation to further enable downstream applications such as WGA and subsequent comparative genomic hybridization (CGH) or massive parallel sequencing.

Regarding CTC isolation, automated micromanipulation gives the advantages of cell imaging, less shear forces and a flexible deposition on various formats (e.g., culture plates, PCR tubes or glass slides). Within the presented study, we focused on the establishment of a robust workflow that combines isolation and downstream processing of single CTCs.

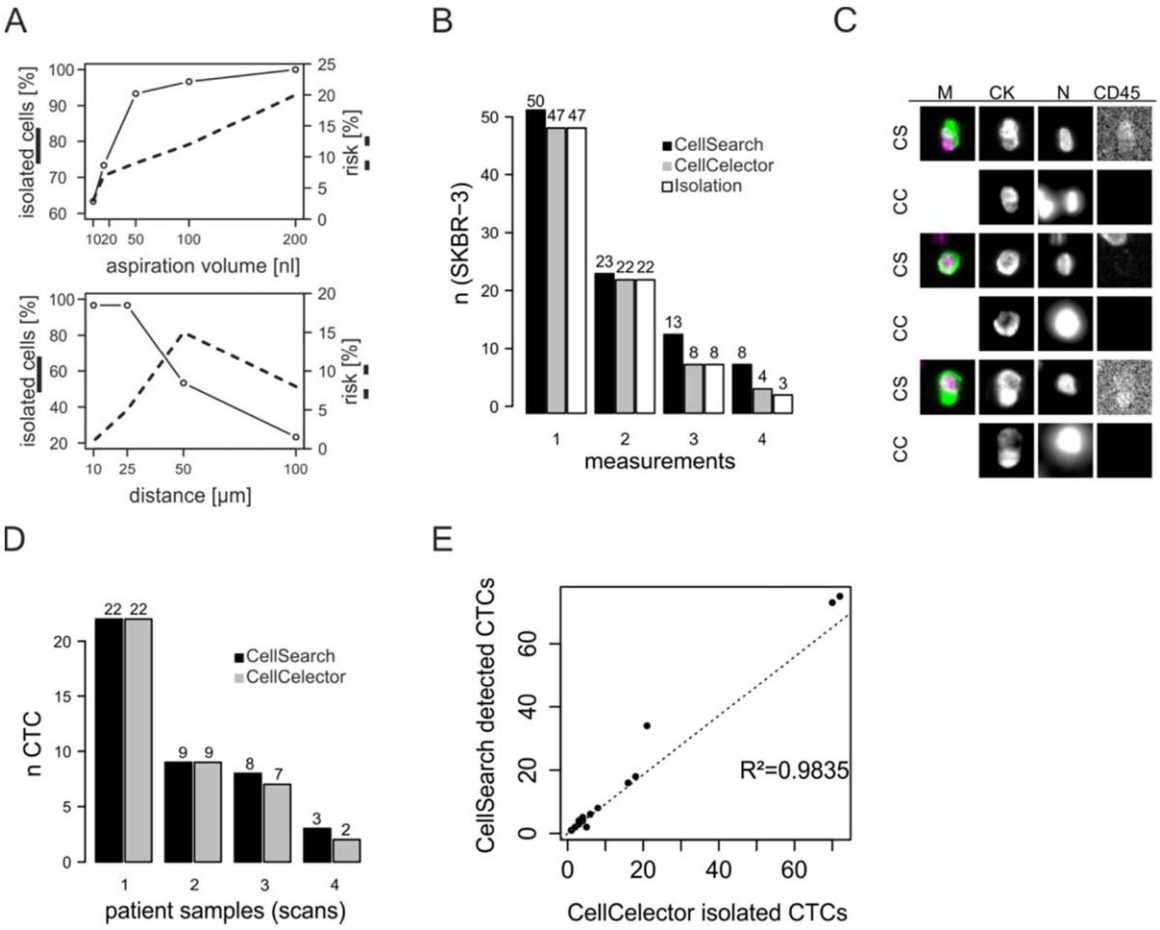

Figure 2. Optimization of single cell isolation with the CellCelector™.

(A) Optimization of picking parameter using SKBR-3 cells led to an optimized aspiration volume of 50 nL at a constant height of 25 µm above the surface. Risk [%] denoted the probability of aspirating unwanted material. (B) CellSearch® SKBR-3 control cells were transferred to a cartridge and were first scanned using the CellSearch CellTracks Analyzer II® (black bar) and then using the CellCelector™ optical system (gray bar). Approximately 95% of cells could be redetected by the CellCelector™ and more than 98% of the identified cells could be isolated after transferring the sample to glass slides (white bar). In 96% a successful deposition was achieved (Isolation/ deposition ratio of 1.04). (C) Representative images of CTCs identified by the CellTracks Analyzer IIR (CS) and the CellCelector™ (CC). (D) Scanning of patient samples. In total, 95% of the CellSearch® identified CTCs (black) were reidentified by the CellCelector™ (gray). (E) Correlation between expected (CellSearch® CTC count) and observed (via CellCelector™) number of CTCs from 20 samples of breast cancer patients (Spearman's rho correlation coefficient $r = 0.98$, $p < 0.00001$). CS = CellSearch, CC = CellCelector, n = number, M = merge, N = nucleus, CK = cytokeratin.

The combination of CTC enrichment and quantification (CellSearch®) with the detection and automated micromanipulation (CellCelector™) represents a powerful and easy workflow for further comprehensive characterization of CTCs.

The herein presented workflow (Figure 1) for single cell isolation from CellSearch® cartridges started with 7.5 mL patient blood which was processed using the CellSearch® system and provided CTC kits. Afterward, the samples were scanned with the CellTracks Analyzer II® and CTC counts were determined. Cartridges with at least one CTC were either processed immediately with the CellCelector™ system or stored at 4°C in the dark until further use. Once the sample has been transferred onto a glass slide, a magnetic field was generated with a commercially available magnet adapter placed beneath the glass slide. Thereby, the ferrofluid-coated cells were attracted to the glass surface positioning them in a single focus layer to facilitate scanning, recording and analysis of the samples. Recorded particle lists were filtered according to the following parameters: positive DAPI and TRITC (CK) signals, a negative signal for CD45-specific labeling, a particle diameter of 4–40 µm, and a gray value mean (fluorescence intensity, color depth) of at least 2000 bpp (bit per pixel) for TRITC. After a manual verification, particles of interest were selected for automatic isolation. Successfully isolated CTCs are deposited in PCR tubes and processed for WGA.

Single cell identification and isolation from one sample using the CellCelector™ can be performed by an experienced operator within 1–2.5 h, with cell identification being the most time-consuming part. Sample quality highly varied from patient to patient and affected the isolation process. For instance, in rare cases cells can be fairly sticky, an effect which can be minimized by placing the sample in EDTA-containing PBS buffer. Additionally, the provided magnetic adapter (for placing glass slides into the microscope) can be precooled minimizing the adhesion of cells to the glass surface. In rare instances, we also observed an improper detection of CTCs due to the formation of cell aggregates or cell debris. However, the workflow was optimized in regard to CTC isolation and deposition rates using the CellCelector™ (both over 95%). Following downstream applications are numerous, such as multigene sequencing, as performed here, or array comparative genome hybridization (aCGH) and—in case of nonfixed samples—culturing of CTCs is also feasible.

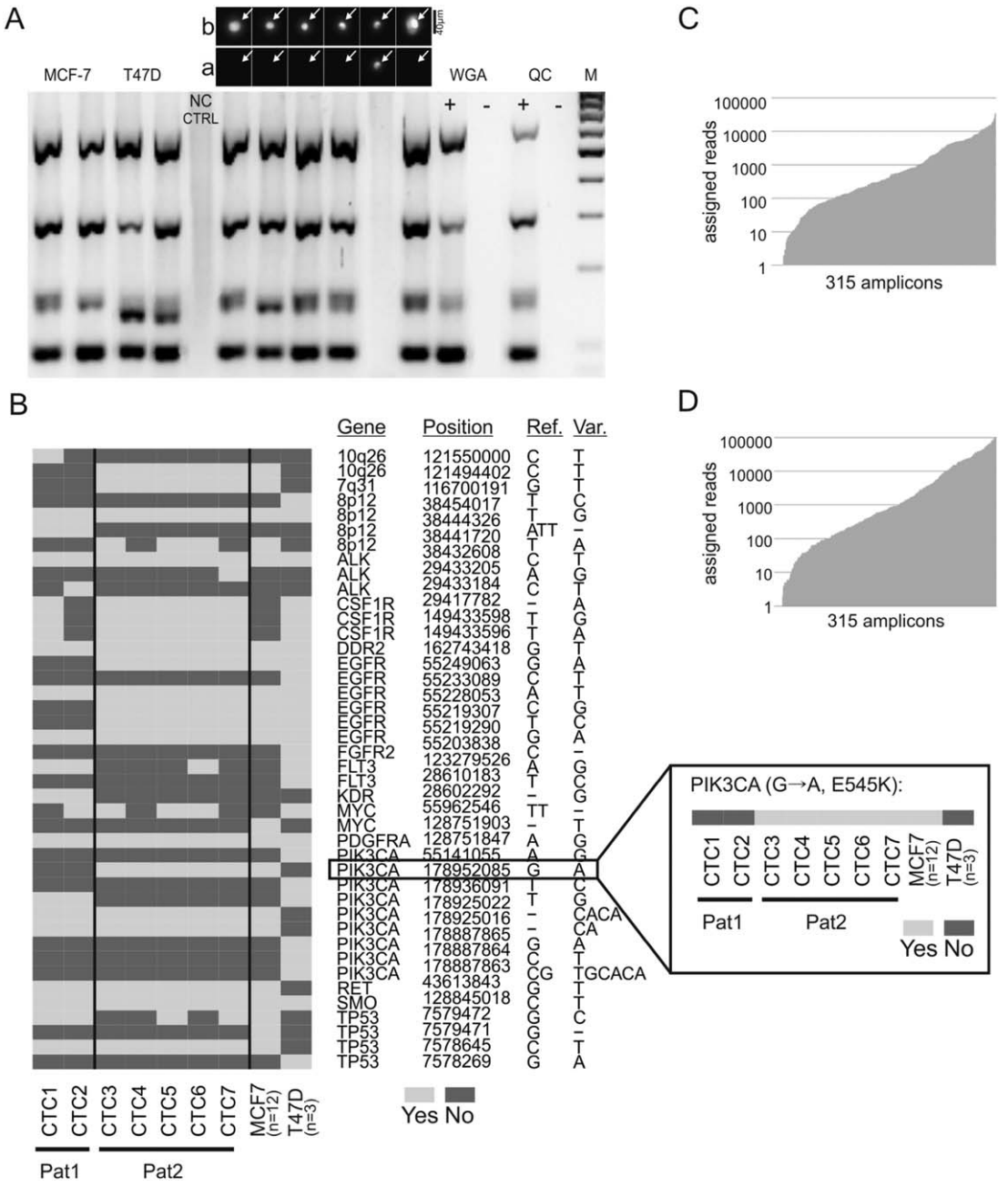

Figure 3. Molecular downstream Analysis of CTCs 467 isolated with the CellCelector™.

(A) As proof of principle single cell isolation and sequencing of MCF-7 and T47D cells were performed. First MCF-7 and T47D cells as well as culture medium (no cell control, NC CTRL) were isolated using the CellCelector™ and the whole genomic material was amplified. A control of the WGA was always performed (WGA +/−). Images of isolated CTCs (patient sample 2) are shown above (b = before/a = after isolation). All single cells showed 4/4 bands, cells with less than four bands are not shown and were not selected for sequencing. WGA and quality control of the WGA process are presented on the right side. WGA products of isolated CTCs were further processed by massive parallel multigene sequencing. 476 a = after CellCelector isolation, b = before CellCelector isolation, M = marker, QC = quality control, WGA = whole genome amplification. (B) Heat map of detected SNPs in T47D, MCF-7, patient sample 1 as well as patient sample 2. All single cells share 7 SNPs. In total, 12 single MCF-7 cells and 3 single T47D cells were sequenced and the results were pooled in silico. Comparison of detected SNPs between patient samples 1 and 2 shows that 10 SNPs are appearing in both patients. Light gray indicates a positive event (SNP), dark gray indicates no event (no SNP). The gene names are listed left side with the respective positions within the gene as well as wild-type nucleotide (Ref.) and mutated form (Var.). Box on the right panel highlights the functional point mutation within the *PIK3CA* gene (E545K) of detected CTCs and cell culture cells. (C) Representative amplicon profile of a single MCF-7 cell. (D) Representative amplicon profile of a single CTC from patient 1.

Optimization of single cell isolation with the CellCelector™

First, appropriate picking parameters were determined (Figure 2A). For this, different aspiration volumes as well as capillary heights and the isolation success were tested using SKBR-3 cells. Additionally, the risk of aspirating unwanted material applying these parameters was recorded to optimize single cell isolation. The optimal aspiration volume was 50 nL with a constant distance between the capillary and the glass slide of 25 µm. By applying these optimized parameters, the risk of aspirating unwanted material was low ($\leq$9%) for cell densities of 25–50 cells/1 mm^2. Detection, isolation, and deposition ratios (Figure 2) were determined using dilution series of fixed and prestained CellSearch® SKBR-3

Table 1. Sequence Coverage of Single Cells

Coverage	>10× (%)	>20× (%)	>100× (%)
MCF7	86	82	63
	90	89	74
	85	83	68
	92	90	76
	90	87	71
	86	80	58
	88	84	62
	89	83	60
	84	81	57
	92	90	78
	86	83	78
T47D	92	91	80
	91	90	84
	87	84	75
Pat 1	88	85	69
	90	89	69
	93	91	69
	90	88	72
	91	89	75
Pat 2	84	82	73
	97	97	93

control cells: 50 SKBR-3 cells and three 1:2 sequentially diluted samples were transferred into CellSearch® cartridges and cells were quantified in four independent measurements via the CellTracks Analyzer II®. Therein, 50, 23, 13, and 8 SKBR-3 cells could be counted. After transferring these samples onto glass slides 47, 22, 8, and 4 SKBR-3 cells could be reidentified with the CellCelector™ (Figure 2B) and all detected cells were successfully isolated by micromanipulation. Out of these cells, 96% were deposited individually on glass slides.

In summary, the results demonstrate that by using the CellCelector™ system single CTCs can be detected, isolated and individually deposited with high recovery rates.

Retrieval of CTCs. Subsequently, we verified whether the standard criteria used to classify CTCs with CellSearch® comprising a minimum diameter of 4 μm, a nucleus visible within the cytoplasm, a round to oval morphology, and $DAPI^+/CK^+/CD45^-$ staining profile are also useful within the CellCelector™ imaging unit. To that aim, CTC images taken by the CellTracks Analyzer II® were compared to images recorded from the same samples with the CellCelector™. In Figure 2C, representative images of patient CTCs taken with CellSearch® and the CellCelector™ are shown in direct comparison. They indicate that CK signals of reidentified CTCs are highly similar indicating appropriate settings for the CellCelector™ imaging unit. In total, 95% of CTCs detected by CellSearch® could be reidentified with the CellCelector™ (Figure 2D). Moreover, almost all cells could be successfully isolated. We determined a correlation of $R^2 = 0.98$ (Spearman's rho correlation, $n = 20$, $p < 0.00001$, Figure 2E) for CTC counts detected by the CellSearch® ($n = 271$) and the CellCelector™ ($n = 252$). The slight discrepancy in CTC counts can mainly be explained by a cell loss during the transfer of CellSearch® cartridge contents onto glass slides. Additionally, detection efficiency is dependent on the quality of the blood sample itself: blood of poor quality might evince more cell debris and clump-like aggregates that do not only hamper pipetting, but also affect the detection of fluorescence signals via the CellCelector™. Regarding deposition efficiency, the success rate was dramatically increased when prefilling the capillary with PBS (10- to 100-fold of aspiration volume) prior to cell picking—thereby applying a higher pressure on the aspirated cell for retrieval.

Taken together, the CellCelector™ fluorescence microscope/micromanipulator system enables highly efficient isolation (picking) and deposition of single CTCs. Apart from that, success of handling and fluorescence and morphologic characteristics for each cell are documented.

CTCs isolated with the CellCelector™ are of high quality

As proof of concept, we isolated breast cancer cell lines (MCF-7 and T47D) as well as CTCs from two different patients. After successful picking of cells (Figure 3Aa and Ab), single genomes were amplified prior to massive parallel multigene sequencing. A subsequent quality control resulted in high-quality DNA (4/4 bands) for 30.6% of all generated WGA libraries (34 out of 111; data not shown). As expected, QC-PCR for the no cell control (NC CTRL) did not generate a signal indicating the absence of contaminating DNA in the aspiration buffer. For downstream application, only samples of high-quality (displaying four amplicons in the QC-PCR) were used (Figure 3A). Besides single cells from cell lines, seven CTCs from two patients were subjected to massive parallel multigene sequencing. Although our data are in accordance with those reported by Polzer et al.,[13] the proportion of single cells generating high quality DNA was relatively low. This might indicate that some cells/CTCs have undergone apoptosis, were damaged, or the WGA procedure itself (e.g., cell lysis, *Mse*I restriction or PCR amplification) was inefficient/not successful. Further, Polzer et al. have shown that cell fixation substantially decreased the percentage of high-quality DNA samples.[13]

Molecular downstream analysis of isolated CTCs

To test the applicability of genomic DNA of micromanipulated CTCs for molecular analyses, WGA products were further analyzed by massive parallel multigene sequencing. In parallel, amplified genomic DNA from single unfixed MCF-7 and T47D cells was analyzed in the same way and results were compared. In total, we sequenced WGA products from 7 single CTCs, 12 single MCF-7, and 3 T47D cells.

A mean amplicon coverage of 1500× was achieved. Eighty to 91% of the amplicons in case of cell line cells and 82–97% in case of CTCs exceeded a coverage of 20×, which we deemed sufficient to call heterozygous mutations at a single cell level (Table 1). Altogether, 96% of the analyzed amplicons had a mean coverage of >20×. Figures 3C and D show representative assigned reads over 315 amplicons of a single MCF-7 and single CTC cell.

From the raw sequencing data, SNP profiles could be compiled. In total, we identified SNPs in 50 genes. Figure 3B shows the detected SNPs in T47D and MCF-7 cells as well as in CTCs of patient sample 1 ($n = 5$ CTCs) and patient sample 2 ($n = 2$ CTCs). A functional *PIK3CA* SNP (G to A, E545K) in CTCs of patient 1, but not in CTCs of patient 2 (Figure 3B, right panel) was detected. The list of all identified SNPs in each individual cell is presented in the supplemental data (Table S1). For MCF-7 cells we could identify the known *PIK3CA* SNP (E545K in chromosome 3, exon 9). In order to calculate the total DNA quality of the patient sample, WGA and sequencing results for CTCs should be compared to those for leukocytes from the same patient.

Via combining the advantages of both systems (CellSearch® and CellCelector™) an easy and reliable workflow applicable for a routine sample processing was established. In the future, we want to apply this powerful method to isolate $EpCAM^{pos}$ and $EpCAM^{neg}$ CTCs, and analyze them by either aCGH or massive parallel multigene sequencing to elucidate the biological nature of these CTC subpopulations.

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Literature Cited

1. Ashworth TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Med. J.* 1869; 14:146–149.
2. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat. Rev. Cancer* 2009;9: 274–284.
3. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LWMM, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med.* 2004;351: 781–791.
4. Giuliano M, Giordano A, Jackson S, De Giorgi U, Mego M, Cohen EN, Gao H, Anfossi S, Handy BC, Ueno NT, Alvarez RH, De Placido S, Valero V, Hortobagyi GN, Reuben JM, Cristofanilli M. Circulating tumor cells as early predictors of metastatic spread in breast cancer patients with limited metastatic dissemination. *Breast Cancer Res.* 2014;16:1–9.
5. Barradas AMC, Terstappen LWMM. Towards the biological understanding of CTC: Capture technologies, definitions and potential to create metastasis. *Cancers* 2013;5:1619–1642.
6. Neves RPL, Raba K, Schmidt O, Honisch E, Meier-Stiegen F, Behrens B, Möhlendick B, Fehm T, Neubauer H, Klein CA, Polzer B, Sproll C, Fischer JC, Niederacher D, Stoecklein NH. Genomic high-resolution profiling of single CKpos/CD45neg flow-sorting purified circulating tumor cells from patients with metastatic breast cancer. *Clin. Chem.* 2014; 60:1290–1297.
7. Fabbri F, Carloni S, Zoli W, Ulivi P, Gallerani G, Fici P. Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs. *Cancer Lett.* 2013;335:225–231.
8. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AGJ, Uhr JW, Terstappen LWMM. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.* 2004;10:6897–6904.
9. Choi JH, Ogunniyi AO, Du M, Du M, Kretschmann M, Eberhardt J, Love JC. Development and optimization of a process for automated recovery of single cells identified by microengraving. *Biotechnol. Prog.* 2010;26:888–895.
10. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucl. Acids Res.* 2010;38:e164.
11. Robinson J, Thorvaldsdóttir H, Winckler W, Guttman M, Lander E, Getz G, Mesirov J. Integrative genomics viewer. *Nat. Biotechnol.* 2011;29:24–26.
12. R Core Team. R: A language and environment for statistical computing. http://www.R-project.org/: R Foundation for Statistical Computing, Vienna, Austria; 2014.
13. Polzer B, Medoro G, Pasch S, Fontana F, Zorzino L, Pestka A, Andergassen U, Meier-Stiegen F, Czyz ZT, Alberter B, Treitschke S, Schamberger T, Sergio M, Bregola G, Doffini A, Gianni S, Calanca A, Signorini G, Bolognesi C, Hartmann A, Fasching PA, Sandri MT, Rack B, Fehm T, Giorgini G, Manaresi N, Klein CA. Molecular profiling of single circulating tumor cells with diagnostic intention. *EMBO Mol. Med.* 2014;6: 1371–1386.

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