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TECHNICAL NOTE

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Implementing microwell slides for detection and isolation of single circulating tumor cells from complex cell suspensions

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Abstract

Cell loss during detection and isolation of circulating tumor cells (CTCs) is a challenge especially when label-free pre-enrichment technologies are used without the aid of magnetic particles. Although microfluidic systems can remove the majority of "contaminating" white blood cells (WBCs), their remaining numbers are still impeding single CTC isolation, thus making additional separation steps needed. This study aimed to develop a workflow from blood-to-single CTC for complex cell suspensions by testing two microwell formats. In the first step, different cell lines were used to compare the performances of Sievewell™ 370 K (TOK, Japan) and CellCelector™ Nanowell U25 (ALS Automated Lab Solutions, Germany) slides for cell labelling and single-cell micromanipulation. Confounding levels of auto-fluorescence inherent to different plastic materials used to cast the microwells, staining recovery rates, and cell isolation rates were determined. In the second step, three different blood preservation tubes were tested for RNA analysis. Lastly, the established workflow was applied to isolate CTCs from peripheral blood samples obtained from metastasized breast cancer (mBC) patients for single-cell DNA and RNA analysis. The detection of CTCs in Sievewell slides profit from better signal-to-noise ratios in the fluorescence channels mainly used for CTC detection. In addition, due to its design, Sievewell supports direct in situ CTC labelling, which minimizes cell loss and leads to single-cell recovery rates after staining of approx. 94%. Detection of PIK3CA mutations in single CTCs verified the applicability of the workflow for the analysis of genomic DNA of CTCs. Furthermore, combined with blood preservation up to 48 h at room temperature in LBguard tubes, panel RT-PCR transcript analysis was successful for single cell line cells and CTCs, respectively. The combined use of Sievewell microwell slides and CellCelector™ automated micromanipulation system improves single CTC detection, labelling and isolation from complex cell suspensions. This approach is especially valuable when samples of high cellular content are processed.

KEYWORDS
CellCelector, circulating tumor cell, microwells, single cell, transcriptomic analysis.

1 | INTRODUCTION

With the development of a plethora of technologies for detection and isolation of circulating tumor cells (CTCs), research on their genotypic and phenotypic features has made significant progress [1–5]. Circulating tumor cells represent tumor cells with heterogeneous cytomorphology and molecular characteristics, which are shed from tumor or metastasis tissues into the body and can be found in body fluids such as the blood [6]. Multiparametric analysis of CTCs is a prerequisite to establish them as clinically relevant liquid biopsy (LB) and a promising non-invasive approach to obtain real-time information on cancer disease in high resolution. In this way, LB may complement the shortcomings of current therapeutic cancer management, which focuses on genotypic and phenotypic analysis of primary tumor tissue or biopsies of metastases and thereby is often not sufficient to display full intra- and inter-tumor heterogeneity.

Different clinical studies have confirmed that the quantification of CTCs is of prognostic relevance in early-stage breast cancer (BC) [7, 8] and metastatic BC (mBC) [9, 10], but their predictive significance to support therapy selection is still unresolved. To improve this situation, there is a need to analyze and understand the CTCs' biology, which—due to cellular heterogeneity, shall be accomplished in single-cell resolution [11, 12] Despite the presence of many methods and technologies to detect and isolate CTCs from blood, the presence of contaminating WBCs still remains an issue to obtain pure single CTCs. This situation is exacerbated when blood volumes larger than the standard 7.5 to 10 ml are investigated to increase positivity rates and CTC numbers, e.g. in cancer patients who are suffering from early stage disease. In order to realize such approaches, pre-analytic workflows have to be created, which avoid multiple purifications and the ensuing incremental CTC loss and provide optimized detection, separation and isolation of single CTCs.

We address this problem here and present a workflow—from blood-to-single CTC—consisting of microfluidic cell separation, labelling of CTCs in a perforated microwell chip followed by micromanipulation combined with preanalytic cell preservation compatible with single-cell DNA and RNA-analysis.

2 | MATERIALS AND METHODS

2.1 | Blood samples

Blood was obtained from healthy donors and mBC patients recruited for the in-house "Augusta study," approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (Ref-No: 3430). All persons gave their informed consent to use their blood samples for CTC analysis. Peripheral blood was collected into LBgard® blood tubes (Biomatrica, United States), Transfix/EDTA tubes (Cytomark Ltd., United Kingdom) or EDTA tubes (BD Vacutainer K2E [EDTA] 18.0 mg, BD, United States), stored at room temperature and was processed within 48 h.

2.2 | Cell culture

Breast cancer cell lines (SKBR3, MCF-7 and T47D), originally purchased from American Type Culture Collection (ATCC), were cultured in complete growth medium containing 89% RPMI 1640 Medium (Gibco, United States), 10% Fetal Bovine Serum (Gibco) and 1% Antibiotic Antimycotic (Gibco). The colon cancer cell line (LS174T) was a kind gift from Angle Europe Ltd. (Guildford, United Kingdom), cells were cultured in complete growth medium, containing 89% Eagles Minimum Essential Medium (ATCC, United States), 10% Fetal Bovine Serum (Gibco) and 1% Antibiotic Antimycotic (Gibco). Cell line cells were harvested with StemPro™ Accutase™ Cell Dissociation Reagent (Thermo Fisher, United States). Pre-staining of cells was done with CellTracker™ Green CMFDA Dye (Thermo Fisher) according to the manufacturer's protocol. Cells were counted in a Neubauer counting chamber.

2.3 | Peripheral blood mononuclear cell (PBMC) preparation

Peripheral blood from healthy donors was collected into EDTA tubes, stored at room temperature and processed within 4 h after blood draw. PBMCs were collected in SepMate-50 tubes (Stemcell Technologies, Germany) according to the manufacturer's protocol. Cells were washed twice and finally suspended in 10 ml DPBS (Gibco). The cell number was determined with a Neubauer counting chamber and adjusted to 200,000 PBMCs/ml.

2.4 | Sample processing

Blood samples were processed with the Parsortix® system (ANGLE, United Kingdom) equipped with separation cassettes harboring a 6.5 μm gap and the following program settings PX_C, PX_PF, PX_S99F, PX_H, and PX_C. Harvested cells were separated onto prepared microwell slides as described below. For spike-in experiments 0.5 ml of 200,000 PBMCs /mL suspensions plus 100 to 500 spiked-in cell line cells were applied.

2.5 | Microwell slides

Sievewell 370 K slides (TOK, Japan. Available from ALS, Germany), hereinafter referred to as Sievewell slides, are microwell array formats. Their main component is a chamber containing a transparent filter composed of approx. 370,000 hexagonal microwells—20 μm in diameter and 25 μm deep. The bottom of each microwell is perforated with 2 microscopic pores—2 μm in diameter, which are connected to a micro-gap situated under the microwell chip and then to two side ports. Pipetting from side ports allows flushing of liquids through the microwells without loss of loaded cells. Before their usage, the slides

are pre-wetted with 100% ethanol (Merck KGaA, Germany) and pre-coated with 2% Bovine serum albumin (BSA) by incubation at room temperature for 30 min according to the manufacturer's instructions. 2% BSA was prepared by dissolving 1 g BSA (Sigma-Aldrich) in 50 ml DPBS (Gibco) and filtered (pore size 0.2 μm; VWR). The cell suspension is pipetted into the chamber and followed by aspiration from the side ports resulting in cells loaded down into the cavities—optimally one cell per cavity. This procedure is supported by washing once with DPBS in combination with negative pressure applied with a 200 μl pipette to discard liquid from slide's side ports.

CellCelector Nanowell U25 slides (ALS, Germany), hereinafter referred to as Nanowell slides, also have a microwell array format. Their main component is a transparent polydimethylsiloxane (PDMS) chip with approx. 200,000 "UFO-shaped" microwells—25 μm in diameter and 25 μm deep. Compared to Sievewell slides, the cavities of Nanowell slides do not have pores at the bottom, so centrifugation at 300 g for 5 min is needed during processing to seed cells or to add the antibody cocktails for cell labeling. Before usage, the Nanowell slides are pre-treated in the same way as Sievewell slides, pre-wet with 100% ethanol and pre-coated with 2% BSA. Then the slides are washed 3 times with 2 ml DPBS. Afterwards, the cell suspension can be loaded onto the Nanowell slide.

2.6 | Autofluorescence determination

Autofluorescence levels of Sievewell slides and Nanowell slides were determined with the fluorescence microscope of the CellCelector™ system. Three fluorescence channels DAPI, FITC, and Cy5 were investigated with exposure times set to 1 ms, 2 ms, 5 ms, 10 ms, 20 ms, 50 ms, 100 ms, 200 ms, 500 ms, 1 s, and 2 s. For each slide, 5 different areas were scanned in 10× magnification. For cells, 50,000 WBCs or SK-BR-3 cells were stained on each slide as described below.

2.7 | Preparation of exact numbers of cells

Cell line cells (SK-BR-3, MCF-7, and LS174T) were pre-labeled with CellTracker™ Green (CellTracker™ Green CMFDA Dye, ThermoFisher) according to the manufacturer's manual. Up to 10,000 pre-labeled cells were distributed onto glass slides and exact numbers of singles cells—between 100 and 200 cells—were separately micromanipulated into Sievewell or Nanowell slides, respectively. Afterwards, both slides were scanned with CellCelector in the FITC fluorescence channel to determine the starting number of deposited cells ("100%" in Table 1).

2.8 | Immunofluorescence labelling in Sievewell or Nanowell slides

To determine staining efficiency and for DNA analysis, cells deposited on both Sievewell and Nanowell slides were fixed with 0.5 ml CellSave™ (Menarini, Italy) reagent for 15 min at room temperature, followed by three times washing each with 2 ml DPBS, Sievewell slides were washed with a 200 μl pipette, all liquid was removed through the pores in each cavity. Fresh DPBS was added to the top of the chamber. Nanowell slides were centrifuged at 300 g for 5 min and then a 1000 μl pipette tip was applied to the corner of the chamber to remove the supernatant and approx. 0.5 ml liquid was left in the slide to cover the cells. Fresh DPBS was filled into the slide by pipetting into a corner, too. Washed cells were incubated with 0.5 ml DAKO protein block (DAKO Agilent, Denmark) at room temperature for 15 min followed by incubation with 0.6 ml antibody staining cocktail containing 0.1% saponin (Sigma-Aldrich) for cell permeabilization, DAPI (F. Hoffmann-La Roche, Switzerland) to stain nuclear DNA, specific antibodies for cytokeratins (clone C11, Alexa Fluor® 488 conjugated, GeneTex, United States) and CD45 (clone 35-ZS, Alexa Fluor® 647 conjugated, Santa Cruz Biotechnology, United States). After incubation for 1 h at room temperature in the dark, both slides were washed 3 times with 2 ml DPBS as described above. Stained cells were kept in DPBS at 2–8°C for up to 48 h until further analysis such as tumor cell detection and single-cell isolation.

For RNA analysis fixation of cells and treatment with DAKO protein block were omitted. Cells were permeabilized by 15 min incubation with 1 ml of 1:10 diluted BD FACS™ lysing solution (BD, United States) at room temperature in the dark, followed by three times washing with 2 ml DPBS as described above. Afterwards, cells were incubated for 30 min with 0.6 ml antibody staining cocktail

TABLE 1 Performances of different microwell slides

			Sievewell	Nanowell	*P*-value[b]
Staining recovery rate		D(μm)[a]			
	LS174T	12	90.35% ± 4.47%	87.34% ± 1.55%	0.332
	MCF7	20	95.05% ± 2.97%	83.58% ± 8.16%	0.084
	SKBR3	24	93.05% ± 3.13%	77.50% ± 3.73%	0.005
	Overall		93.73% ± 2.68%	82.44% ± 6.59%	0.004
Single-cell isolation rate					
	LS174T		89.26% ± 2.32%	94.67% ± 9.24%	0.381
	MCF7		95.55% ± 3.85%	95.92% ± 3.57%	0.909
	SKBR3		89.12% ± 1.26%	98.18% ± 3.15%	0.010
	Overall		91.31% ± 3.95%	96.26% ± 5.42%	0.042

[a]D means the average cell diameter.
[b]*t*-Test.

(DAPI; Cytokeratin [CK], C11 + AE1/AE3, TRITC conjugated, Aczon, Italy; and CD45), followed by three washing steps as described above. Stained cells were immediately scanned and processed with CellCelector™ system (ALS, Germany) for single-cell isolation.

2.9 | Tumor cell detection and single-cell isolation

Tumor cells were detected and isolated with the CellCelector™ system. Slides were placed onto the microscope stage orand a single-cell isolation template was selected in the CellCelector internal software. The template contains automatic user-defined scanning, image analysis, visualization of single candidate tumor cells in a gallery format and single-cell isolation. Scanning was done using DAPI, FITC (or TRITC), and Cy5 fluorescence channels at 10× magnification. The CellCelector imaging software processed scanned images and the cells were scored according to the signal intensities. $DAPI^{pos}$/CK-$FITC^{pos}$ or CK-$TRITC^{pos}$/CD45-$Cy5^{neg}$ cells were considered as tumor cell candidates and selected from the gallery for verification at 40× magnification. Positively identified CTCs were then micromanipulated using glass capillaries with 30 μm diameter at the tip. Photos of before and after picking were automatically documented by the CellCelector internal software(Supplemental Figure 1). In detail, 1 μl DPBS was aspirated into the capillary and the tip of the capillary was placed into the microwell slides to cover the relocated Sievewell or Nanowell cavities. Then, 0.001 μl −0.05 μl liquid together with the tumor cell was aspirated into the glass capillary and deposited into a flat bottom optical PCR tube. Successful deposition was controlled by microscopic inspection. Isolated cells were kept in a − 80°C freezer until further analysis.

2.10 | Single-cell genomic analysis

Patient blood was collected into EDTA blood tubes and processed with the Parsortix system. Afterwards, cells were spread onto Sievewell slides and stained as described, CTCs were subsequently detected and single cells were deposited into PCR tubes for genomic analysis.

For genomic analysis, DNA of single cells was amplified with Ampli1™ WGA Kit (Menarini, Italy) according to the manufacturer's protocol. The quality of amplified DNA was determined by the supplementary Ampli1™ QC Kit (Menarini, Italy). High-quality samples with 3 or 4 PCR bands were eligible for further analysis. Amplified genomic DNA was used for sequencing analysis using a multiplex PCR-based next-generation sequencing-panel established in our lab. [13].

2.11 | Transcriptome analysis

For transcriptome analysis LS174T cells with the previously determined expression profile of a gene panel of interest were used [14, 15]. Cells were pre-labeled and spiked into healthy donors' blood, which had been collected in tubes containing either EDTA (BD), LBgard or Transfix/EDTA and were stored at room temperature for 1 h, 24 h or 48 h, respectively. Samples were then processed with the Parsortix system—as described before—at 99 mbar of pressure [16]. Captured cells were spread onto Sievewell slides and processed as described above. Single cells were deposited into flat bottom PCR-tubes each containing 10 μl lysis buffer for transcriptome analysis following the procedure described by van Strijp et al. [15]. The lysis buffer were made by 0.5 × TBE (Invitrogen, United States), 0.5% (v/v) Triton-X100 (Merck KGaA, Germany), and 2 U/μl RNase inhibitor (RNaseOUT™ Recombinant Ribonuclease Inhibitor, Invitrogen) in DNase/RNase free water (UltraPure™ DNase/RNase-Free Distilled Water, Gibco).

For the CTC analysis, peripheral blood of mBC patients was collected into LBgard collection tubes and stored at room temperature for 24 h, followed by further processing with Parsortix and Sievewell slides as described above.

Lysates of isolated cell line cells and CTCs were processed as follows: isolated cells in lysis buffer were heated for 5 min at 80°C and cooled to 4°C on the BioRad CFX96™ real-time PCR detection system to revert any crosslinking. 5 μl of the RNA extracts was used as input in a 25 μl RT-PCR pre-amplification reaction using SuperScript III Platinum One-Step Quantitative RT-PCR reagents (Invitrogen, ThermoFisher Scientific) on the BioRad CFX96™ system. The following thermal pre-amplification profile was applied: after a cDNA synthesis step at 50°C for 30 min and an initial denaturation step at 95°C for 5 min, 11 PCR cycles were performed consisting of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 4 min.

A total of 2 μl of undiluted pre-amplified DNA product was used as input in a 25 μl qPCR amplification reaction using SuperScript III Platinum One-Step Quantitative RT-PCR reagents (Invitrogen, ThermoFisher Scientific) on the BioRad CFX96™ system. The thermal profile used for the qPCR reactions was as follows: after an initial denaturation step at 95°C for 5 min, 50 PCR cycles were carried out consisting of denaturation at 95°C for 15 sec, primer annealing/extension at 60°C for 45 sec. Fluorescent probe detection was performed at the end of each cycle. Cq values were calculated by means of the second derivative method using an algorithm developed by Philips.

2.12 | Image analysis

Image analysis was performed with CellCelector internal software and ImageJ (Wayne Rasband, National Institutes of Health, United States).

2.13 | Statistical analysis

Statistical analyses were performed using RStudio (http://www.rstudio.com/; RStudio, Inc., Boston, United States) [17]. *P*-values <0.05 were considered as statistically significant. Statistical tests used are indicated in figure legends. Data shown in Figures 1 and 2 was visualized with Graphpad Prism 7 (Graphpad Software, United States).

3 | RESULTS

3.1 | Auto-fluorescence—Sievewell slides perform better than Nanowell slides

To optimize immuno-fluorescence detection of labeled cells, we first determined the auto-fluorescence levels of Sievewell slides and Nanowell slides. These slides were scanned with the CellCelector™ system at different wavelengths and exposure times ranging from 1 ms to 2 s and gray values were determined with ImageJ as a measure of background fluorescence. Then, both microwells were loaded with SKBR3 cells or PBMC cells, and labeled on-chip with DAPI/anti-CK^{FITC}/$CD45^{Cy5}$ staining/labeling cocktail (Figure 1). Afterwards, both microwell slides were scanned again with the CellCelector. In Figure 1B, the signal-to-noise ratios (SNRs) obtained for both microwell slides at different fluorescence channels are depicted.

In the DAPI channel (358 nm), the background gray values of Sievewell slides range from 520 to 15,914, depending on exposure duration. The highest signal-to-noise difference (SND) of 4892 is observed at 1 s exposure (Supplemental Figure 2). For Nanowell slides, the highest SND of 5304 is observed for 200 ms exposure (background ranges: 531 to 16,382). For both slides the highest SNRs are obtained at 100 ms with 2.4 for Sievewell and 2.3 for Nanowell slides, respectively.

In the FITC channel (475 nm), for Sievewell slides the highest SND of 9330 is observed at 2 s exposure (background ranges: 513 to 2740); for Nanowell slides this is 5136 also at 2 s exposure time (background ranges: 512 to 1945). For both slides the highest SNRs are found at 1 s with 6.2 for Sievewell and 4.2 for Nanowell slides.

In the Cy5 channel (604 nm), for both slides the highest SND are observed at 2 s with 2314 (background ranges: 512 to 968) for Sievewell slides and with 790 for Nanowell slides (background ranges: 512 to 927). For both slides the highest SNRs are at 2 s with 2.4 for Sievewell and 1.9 for Nanowell slides.

For both Sievewell slides and Nanowell slides, the CellCelector software and ImageJ was able to distinguish cell signals from background signals at all three tested fluorescence channels (DAPI, FITC, Cy5). Sievewell slides exhibit higher SNDs in the FITC and Cy5 channels while Nanowell slides enable a slightly higher SND in the DAPI channel. However, in all three tested fluorescence channels Sievewell slides exhibit higher SNR than Nanowell slides.

Due to this superior performance—especially in the FITC and Cy5 channels—the use of Sievewell slides is more advantageous for CTC detection.

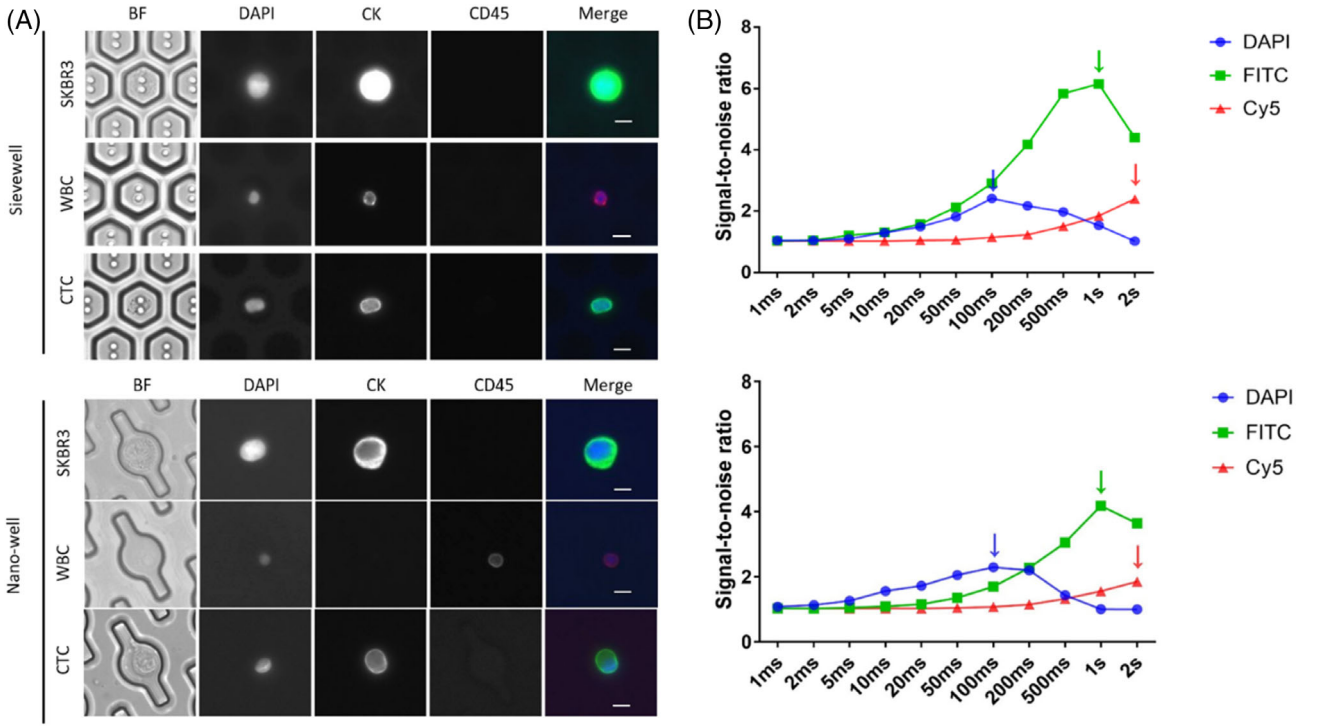

FIGURE 1 Comparative analysis Sievewell slides and Nanowell slides. (A) Capture, staining and immunofluorescence analysis of SKBR3 cells spiked into blood and of CTCs. Depicted are both microwells—3 top lines Sievewell slides, 3 bottom lines Nanowell slides—loaded with SKBR3 cells or CTCs in brightfield (BF), 358 nm (DAPI), 475 nm (FITC) and 604 nm (Cy5). Magnification: 40×, size bar 10 μm.(B) Determination of SNRs. Top graph: Sievewell slide; bottom graph: Nanowell slide. The exposure times range from 1 ms to 2 s. Gray values were measured using ImageJ. The solid lines indicate the signal-to-noise-ratios calculated from gray values obtained for labeled cells and of background fluorescence emitted by the plastic material at tested wavelengths DAPI (blue line), FITC (green line), Cy5 (red line). SKBR3 cells were stained with DAPI and labeled with AlexaFluor 488-conjugated antibodies specific for cytokeratin. WBCs were labeled with AlexaFluor 647-conjugated antibodies specific or CD45. SKBR3 cells were scanned and measured for DAPI and FITC, WBCs were scanned and measured for Cy5. Arrows indicate the optimal SNRs determined for each wavelength. Calculated with Graphpad Prism 7.

3.2 | Single-cell recovery—Sievewell slides perform better than Nanowell slides

Minimal loss of CTCs when applying the detection-isolation workflow is very important. Therefore, we determined staining recovery rates and single-cell isolation success rates for both, Sievewell and Nanowell slides.

3.2.1 | Staining recovery rate

The staining recovery rate is defined as the ratio of cell numbers detected before and after incubation of the cells with the DAPI/antibody cocktail, and is providing a measure for cell loss caused by on-chip staining and washing steps. The obtained results are shown in Table 1.

The mean staining recovery rate for the three cell lines tested was 93.73% (± 2.68%) for Sievewell slides, whereas with Nanowell slides approximately 82.44% (± 6.59%) of cells were found after staining. Looking at the cell sizes a trend for increased cell loss in the Nanowell slides with increasing cell sizes was observed: the lowest recovery rate was achieved with SKBR3 cells, which was significantly lower compared to MCF-7 and LS174T cells (Supplemental Table 1). For the latter two cell lines no significant difference in staining recovery rates was measured. For Sievewell slides, no significant differences in staining recoveries were observed for all three cell lines tested.

3.2.2 | Single-cell isolation rate

The effective isolation of a CTC as single-cell, in view of the high (WBC) cellular background, is an important prerequisite for downstream molecular analysis methods. For both, Sievewell slides and Nanowell slides, the single-cell line cells isolation rates were above 90%, with Nanowell slides (96.26% ± 5.42) being slightly superior to Sievewell slides (91.31% ± 3.95). Additionally, for Nanowell slides the differences in cell sizes did not influence the single-cell isolation success rate, underlining the high performance of the CellCelector system reported before [18]. With Sievewell slides large SKBR3 cells were micromanipulated with significant lower success rates from Sievewell slides compared to Nanowell slides.

3.2.3 | Total recovery rate

When both recovery rates are taken into account the Sievewell slides performed better than the Nanowell slides (85.58% vs. 79.37%).

This result and the observation that CTCs are mostly smaller than SKBR3 cells (Figure 1)—there is no significant difference in single-cell isolation rates but a significant advantage for Sievewell slides regarding staining recovery rates for smaller cells—prompted us use Sievewell slides in further experiments processing breast cancer patient blood samples for CTC isolation.

3.2.4 | Processing breast cancer patient blood samples for CTC isolation

Since "real" CTCs differ in their physical and phenotypic features from cell line cells—one is cell size (Figure 1)—we applied the established workflow to blood samples obtained from 2 patients suffering from luminal mBC to test its performance. Due to the fact that we were ignorant of the real CTC numbers per samples the staining recovery rate could not be determined. The cell suspensions obtained after Parsortix pre-purification were labeled/stained on-chip followed by scanning. A total of five blood samples were processed and approx. 92.4%—in total 145 CTCs—of scanned and identified CTCs could be successfully picked indicating that the workflow allows detection of CTCs and their highly efficient micromanipulation.

3.3 | A workflow for multiplex RT-PCR transcriptome analysis

To optimize a single CTC analysis, preanalytic procedures have to be adjusted to the downstream molecular method. Previous experiments had established that transcriptomic analysis of single LS174T cells was possible when they were isolated form a buffer solution [15]. Therefore, CellTracker Geen pre-labeled LS174T cells were spiked into healthy donors' blood that had been collected in either EDTA, LBgard or Transfix blood preservation tubes. Subsequently, the above established workflow comprising Parsortix pre-purification and Sievewell on-chip staining was applied followed by scanning and micromanipulation of single cells into PCR tubes. Afterwards, a multiplex RT-PCR transcriptome analysis investigating 20 selected transcripts expressed in LS174T cells was executed after storage of the blood samples for 1, 24, and 48 h at room temperature in the indicated preservation tubes (Figure 2 A) [15].

After 1 h "incubation" in EDTA, multiplex RT-PCR was successful in 90% of the tested transcripts. 70% of the transcripts could be amplified after storage in LBgard tubes. Preservation of the cells in Transfix resulted in signals in only 30% of tested transcripts. After 24 h in LBgard, the numbers of successfully amplified transcripts increased slightly to 90%; for preservation in EDTA and Transfix tubes this number dropped to approx. 25% and 15%, respectively. After 48 h at room temperature for the latter two preservation tubes almost no transcript could be detected, while preservation in LBgard tubes still enabled positive PCR results in 90% of the transcripts.

These data suggest that preservation of (tumor) cells in LBgard tubes at room temperature provides good RT-PCR conditions up to 48 h after blood draw. Since this represents a decisive feature for the application in the clinic, we decided to apply LBgard blood collection tubes to the subsequent CTC analysis in mBC patients' blood.

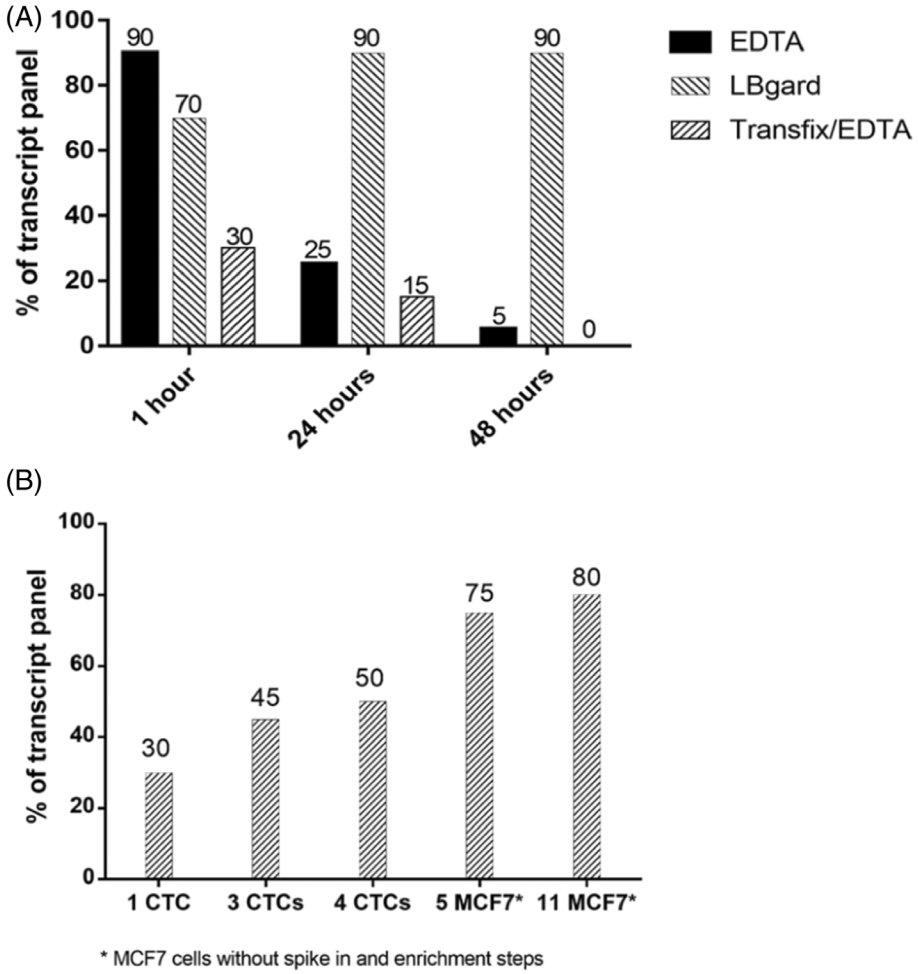

FIGURE 2 RT-PCR transcript panel analysis in single LS174T cells and CTCs. Depicted is the % of detected transcripts of a total panel of 20 transcripts [15]. (A). Analysis of single LS174T cell mRNA evaluation at different time points after blood draw into EDTA, LBgard, and Transfix blood preservation tubes. After preservation for 24 and 48 h in LBgard, single cells scored the highest % of panel transcripts. Calculated with Graphpad Prism 7. (B). Comparative panel analysis of CTCs after 24 h preservation on LBgard. Different CTC counts were analyzed in comparison to MCF-7 cells. (MCF-7 cells have not been spiked into blood and were therefore not processed according to the workflow). Calculated with Graphpad Prism 7.

3.3.1 | CTC analysis in mBC patients' blood

Based on the experience made with spiked LS174T cells, we also tested the established multiplex RT-PCR transcriptome analysis with CTCs donated by an mBC patient harboring many CTCs in her blood. Blood collected in LBgard was processed according to the established workflow (Supplemental Figure 3) and CTCs were isolated. Multiplex RT-PCR transcriptome analysis of single or pooled CTCs was performed resulting in successful amplification of 50% of the transcript panel for a 4-CTCs sample. This percentage dropped to 45% of tested transcripts in a 3-CTCs sample and, to 30% in single CTCs. Since this transcript panel had previously been established to investigate colon adenomas and carcinomas the expression of the transcript panel was tested in unprocessed MCF-7 cells and compared to the percentages of detected transcripts in the CTCs. In the RT-PCR analysis, 75% of transcripts were amplified from a pool of 5 unprocessed MCF-7 cells, 80% of the transcripts were successfully amplified from 11 unprocessed MCF-7 cells, respectively. In relation to the "maximum expectable expression levels" observed in the unprocessed MCF-7 cell pools (75–80%) 37–40% and 62–67% of the transcripts were identified in 1 CTC and 4 CTCs, respectively.

3.4 | A workflow for nucleic acid analysis of single CTCs

We also tested the applicability of Sievewells for genome analysis using 15 single CTCs from the same mBC patient and processed them according to the established workflow. The patient blood was collected use EDTA tubes. After libraries of genomic DNAs had been generated by WGA, we obtained high-quality WGA products - 3 bands or 4 bands of quality control PCR - for 5 CTCs (33.3%). These products were PCR-amplified and sequenced with the MiSeq system to detect *PI3CA*, *ESR1*, *AKT1*, and *ERBB2* hotspot mutations [13]. In two cells the *PIK3CA* hotspot H1047L mutation in exon 21 was found at a 99% and 49% variant allele frequency (VAF), respectively. One cell harbored the PIK3CA mutation W552C in exon 10 at a VAF of 29%. In the other two CTCs no mutations were found.

The influence of LBgard preservation for 24 h on the quality of nucleic acids was tested with T47D cells. They were spiked into whole blood sampled in LBgard and stored for 24 h at room temperature. The sample was then processed according to the established workflow. Cell enrichment was achieved with the Parsortix system (6.5 μm cassette and the S99F program), the trapped cells were then spread onto a Sievewell slide and stained as described. Labeled cells

were subsequently detected and isolated in the CellCelector system. Ten single cells were micromanipulated into PCR tubes for genomic DNA analysis. DNA of single cells was amplified with Ampli1™ WGA Kit. The quality of amplified DNA was determined by the supplementary Ampli1™ QC Kit (Menarini, Italy; Supplemental Figure 4).

4 | DISCUSSION

Detection and isolation of CTCs in peripheral blood is like finding the "needle in the haystack". The current state-of-the-art to detect and enumerate CTCs with the CellSearch system is using ferrofluid to capture CTCs and to immobilize them by magnetic force during staining and washing processes to the wall of the plastic incubation tube to reduce cell loss [19, 20]. However, when detection methods are used where the cells of interest are not bound to ferrofluid or magnetic beads, cell loss during the staining process can be quite substantial, for example, when using microfluidic systems. One of these systems, the Parsortix system is able to deplete up to 99.99% of WBCs from a blood sample still leaving a high number of contaminating cells in the CTC-containing cell suspension, making further CTC purification steps inevitable for high-quality single-cell analysis [21]. Besides, there may be the need to use, for example, filtration systems with smaller pores in order to isolate cells of smaller sizes, in this way also the number of co-isolated WBCs would increase [22]. However, further handling steps will not only cause higher cell loss but will also extend the processing time accompanied with the risk of acerbated degradation effects confounding, for example, gene expression analysis. We therefore set out to test and implement microwell format plates into a workflow from "blood-to-single-CTC" comprising in situ cell labeling, washing, scanning and single-cell micromanipulation to avoid cell loss and to reduce hands-on time during CTCs detection and isolation.

We took advantage of two microwell slide formats—Sievewell slides and Nanowell slides—each containing approx. 370,000 and 200,000 cavities per chip, respectively, to trap single cells. First, we tested the auto-fluorescence levels emitted by the slides' plastic materials at the main fluorescence channels used for CTC detection (DAPI, FITC, Cy5). Although both slides have quite high background fluorescence levels, the imaging software implemented into the CellCelector system, which was used for single-cell micromanipulation, or ImageJ can discriminate between labeled cells' fluorescence signals and background fluorescence emission. At all three tested fluorescence channels Sievewell slides outperformed Nanowell slides with higher SNRs. Besides, the higher SNRs in FITC and Cy5 channels observed for labeled cells in Sievewell slides resulted in improved image qualities compared to Nanowell slides (Figure 1A) making them more appropriate than Nanowell slides for CTC detection as CTCs are mostly discriminated from WBCs with AlexaFluor 488 conjugated pan-CK-antibodies and AlexaFluor 647-conjugated anti-CD45 antibodies. The fact that identical antibody cocktails and protocols—except for way the cells are washed on each chip—were used is pointing toward a less efficient labelling of cells in Nanowell slides compared to Sievewell slides. Staining with DAPI does not seem to be as critical.

4.1 | Staining recovery rate—reduced cell loss in Sievewell slide

To avoid cell loss, high cell recovery rates are very important. The staining recovery rate for the deposited cells was in total approx. 11% higher for Sievewell slides than for Nanowell slides (93.73% vs. 82.44%). This difference was statistically significant for each tested cell line and was mainly caused by the increased loss of the larger SKBR3 cells during staining in the Nanowell slides. This fits into a trend for higher cell loss in the Nanowell slides with increasing cell size and becomes significant between the smallest LS174T cells (12 μm) and the twice as large SKBR3 cells (24 μm) (Supplemental Table 1). No such trend exists with Sievewell slides. This may be explained with their special design: each microwell contains two microscopic pores of 2 μm diameter perforating the cavities' floor which allow liquid to pass through thereby holding cells in the cavity. Nanowell slides do not have this filter function, which is why any liquid has to be aspirated from the top of the slides. This may cause turbulence, flushing cells out of some cavities, even if pipetting is performed very carefully. Another reason for cell loss in Nanowell slides may be the need to centrifuge the cells into the cavities. Due to their pores Sievewell slides do not have to be centrifuged. Consequently, less handling is needed and cells are treated more gently reducing both cell loss and cell damage. In theory, Sievewell slides could provide cell recovery rates close to 100%, but due to the presence of corners in their current design, some cells are trapped at the outer edges of the chip, excluding them from being scanned or properly detected.

4.2 | Single-cell isolation rate—high in both Sievewell and Nanowell slides

Both, Sievewell slides and Nanowell slides, achieved single-cell isolation rates above 90%, with Nanowell slides being significantly ($P \leq 0.042$) superior to Sievewell slides. For SKBR3 cells, the single-cell isolation rate is significantly better with Nanowell slides than with Sievewell slides. This may be the result of smaller diameter of the cavities, which is 20 μm for Sievewell slides and 25 μm for Nanowell slides. As a consequence, the SKBR3 cells may not perfectly fit into the cavities thereby making single-cell isolation more difficult. Indeed, we observed that sometimes more than one SKBR3 cell was picked when tumor cells were located in neighboring cavities. In addition, the distances between the cavities are smaller in Sievewell slides than in Nanowell slides(Supplemental Figure 1). Despite this, Sievewell slides exhibit a better total recovery rate over all cell lines than Nanowell slides (85.58% vs. 79.37%), which mainly results from significantly better staining recovery rates. These compensate for lower single-cell isolation rates achieved with Sievewell slides for LS174T cells and SKBR3 cells. This observation becomes overt when comparing the total recovery rates per cell lines: for LS174T cells it was 80.64% versus 82.68%, for Sievewell and Nanowell slides, respectively; for MCF-7 cells, it was 90.82% versus 80.17%; for SKBR3 it was 82.92% versus 76.09%.

From these data, we conclude that Nanowell slides may be advantageous in situations when cells shall only be micromanipulated without prior staining. Also for Nanowell slides different cell sizes did not influence the single-cell isolation success rate underlining the high performance of the CellCelector system reported before [18]. Yet, as soon as cells have to be stained on-chip followed by micromanipulation Sievewell slides should be used, which is why they were implemented into a complete workflow "from blood-to-single-cell analysis."

4.3 | Preservation of blood in LBgard enables nucleic acid analysis in single cells

Testing pre-analytic parameters is highly important for successful single-cell analysis—a topic emphasized by activities performed, for example, in Cancer-ID (http://www.cancer-id.eu). This large research consortium embarked on harmonizing SOPs and protocols for CTC isolation, preparation and analysis [16, 23]. Albeit on a much smaller scale, we compared three blood preservation tubes—EDTA tubes, LBgard tubes and Transfix/EDTA tubes—and tested their impact on downstream nucleic acid analysis of CTCs isolated with the established workflow. In combination with a panel RT-PCR preservation of blood in LBgard enabled a more or less constant transcript detection rate between 80% and 90% in isolated single LS174T cells. Transfix/EDTA tubes have been designed to preserve cell surface antigens for improved labeling with antibodies and to enable single-cell DNA analysis [24–26]. Only limited data are available regarding the compatibility of Transfix/EDTA with downstream mRNA quantification. In our hands-already when immediately processing Transfix/EDTA preserved blood only 30% of selected transcripts were detected. This ratio decreased even more after 24 and 48 h, which is surprising but is in line with data recently published by Baird et al. [27]. TransFix/EDTA allowed these authors to store samples between 24 and 120 h at room temperature before analysis and treated cells could be used for downstream gene analysis. However, a pretreatment step was carried out, which increased mRNA yield 100× over levels obtained with untreated TransFix/EDTA samples. The authors argue that fixatives in the TransFix tubes protect cellular RNA from complete degradation. However, fixed RNA is not compatible with reverse transcription for RT-qPCR analysis [28]. To address this, a sample pretreatment step was adopted to reverse formaldehyde-fixed RNA to native species without apparent RNA hydrolysis. The most successful performance was observed in dilute Tris, phosphate, or similar buffers at 70°C for 30 min [28]. In our protocol isolated cells in lysis buffer were heated for 5 min at 80°C and cooled to 4°C.

LBgard tubes were primarily developed for cell-free DNA (cfDNA) analysis. The tubes contain a proprietary reagent that stabilizes whole blood preventing the release of contaminating cellular genomic DNA (cfDNA). However, it seems that LBgard tubes not only stabilize cfDNA but also CTCs and WBCs outperforming EDTA and Streck tubes. In our hands the use of LBGard outperformed EDTA and Transfix in preserving CTCs for RNA analysis. Regarding permeabilization, the FACS lysing solution (FLS) which is a mild fixation and permeabilization reagent containing diethylene glycol, formaldehyde, and methanol, was compared with Saponin. From LS174T cells, which were spiked into blood and processed following the whole workflow, 80% of investigated transcripts were recovered with FLS, 55% with Saponin. Therefore, we selected FLS permeabilization for our RNA analysis.

5 | LIMITATIONS

As with most CTC analysis performed in a single-center CTC sample size is very low and should be increased to validate the workflow. But despite this issue in both DNA and RNA analysis of CTCs our results indicate that the described workflow is suitable for nucleic acid analysis of CTCs detected and isolated from complex cell suspensions and that it merits to be tested in larger sample cohorts for further optimization. Next-generation sequencing should be used to test the complexity of DNA and RNA libraries of cell obtained with the established workflow as our analyses can only be considered as proof-of-principle description. Sticking to peripheral blood samples in such a trial, though, will be very time-, cost- and sample-intensive. Late developments in the CTC-field are pointing toward the benefit of focusing either on few informative cancer patients with high CTC numbers or on applying innovative technologies such as Diagnostic Leukapheresis [22, 29–31] to increase the volume of blood screened for CTCs. The herein established workflow may not only be verified within a trial employing such concepts but may also be further refined.

6 | CONCLUSION

The combination of LBgard blood preservation tubes, Parsortix for blood separation, Sievewell microwell chips for in situ labeling and micromanipulation of CTCs using CellCelector enables high recovery rates of CTCs from complex cell suspensions for downstream DNA and RNA analysis.

AUTHOR CONTRIBUTIONS

Liwen Yang: Conceptualization (lead); investigation (lead); writing – original draft (lead). **Mahdi Rivandi:** Resources (supporting). **André Franken:** Methodology (supporting); writing – review and editing (supporting). **Maarten Hieltjes:** Methodology (supporting). **Pieter Jan van der Zaag:** Funding acquisition (equal); writing – review and editing (supporting). **Constantin Nelep:** Funding acquisition (supporting); writing – review and editing (supporting). **Jens Eberhardt:** Funding acquisition (supporting). **Stefan Peter:** Funding acquisition (supporting). **Dieter Niederacher:** Project administration (supporting). **Tanja Natascha Fehm:** Supervision (supporting). **Hans Josef Neubauer:** Project administration (lead); supervision (lead); writing – review and editing (supporting).

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CONFLICT OF INTEREST

M.H. and P.J.Z. were employees of Philips Research Laboratories; C.N. is an employee of ALS Automated Lab Solutions GmbH; J.E. is the CEO of ALS Automated Lab Solutions GmbH; S.P. is an employee of ANGLE Europe Ltd.

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SUPPORTING INFORMATION

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