Dissertation

CIRCULATING TUMOR CELLS AS BIOMARKER FOR MINIMAL RESIDUAL DISEASE IN PROSTATE CANCER

submitted by

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There are only two ways to live your life.

One is as though nothing is a miracle.

The other is as though everything is a miracle.

--- Albert Einstein

This thesis is sincerely dedicated to my beloved parents,

my loved husband, Dr. rer. nat. Lei Shi,

AND my lovely son, Gezhi Shi (Youyou).

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Graz has become a special **HOMETOWN** for me and my families, as it is the place of my son's birth!

DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

The second part of the result in this thesis has been published as follows:

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Shukun CHEN

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SUMMARY

Circulating tumor cells (CTCs) are released by primary tumor lesions or metastases into peripheral blood. Recently CTCs have received substantial attention because of their potential benefits for detecting cancer cell dissemination and influencing the decision for treatment. However, technologies enabling CTC detection and isolation are challenged by its extreme rarity in the blood stream, which hampers the application in clinical routine. Among many recently developed techniques, the CellCollector DC01 (DC01, GILUPI GmbH, Potsdam, Germany), a medical wire functionalized with anti–epithelial cell adhesion molecule (EpCAM) antibodies, is a CE certified medical device already in clinical application. Unlike other methods which use limited volumes of blood as starting material *ex vivo*, the DC01 allows isolating CTCs directly from the peripheral blood circulation.

Up to now, most of the studies of CTCs have been performed in metastatic cancers. Its usefulness to be an early prognostic marker in detection of minimal residual disease in patients with localized primary tumors is still unknown due to its even lower concentration in the blood, which requires advanced techniques with high sensitivity and specificity. **In the first part of the result in the current thesis** (Chapter 3), 51 samples from high-risk prostate cancer (PCa) patients without metastatic setting were included for CTC detection and enumeration using two different methods, CellSearch system and DC01. **The aims of this part of study are**: 1) to compare the analytic performance of different CTC detection platforms for clinical use in high-risk PCa patients; 2) to evaluate that whether the measurement of CTC level is useful in predicting relapse and responses to the therapy (surgery & radiotherapy) in early stage of PCa; 3) to improve the CTC detection in early-stage cancer disease with high sensitivity and specificity using combined methods.

Our preliminary data show that DC01 report almost twice as many patients to be CTC-positive (39.2%, 20 of 51) as compared to the CellSearch (19.6%, 9 of 46) with higher numbers of CTCs per patient detected by DC01 (range = 0-15; median = 0; 75th percentile = 1) than by CellSearch (range 0-5; median = 0; 75th percentile = 0) before undergoing radiotherapy. Paired analysis across all 86 samples (i.e. before and after therapy) showed DC01 to detect higher CTC counts than

CellSearch (P = 0.0062). Statistical analysis did not reveal a correlation between CTC positivity and any of the pathological features, including PSA level, Gleason score, and T stage.

Moreover, when it comes to personalized medicine, analyses exclusively based on CTC enumeration are rather insufficient. CTCs detected by DC01 cannot be recovered for the purpose of single cell analysis. Hence, a newly developed approach is introduced and we aim at retrieving captured CTCs for downstream single cell analysis. This novel so-called Catch and Release detector [CellCollector type Detektor CANCER03 (Catch & Release, C&R, GILUPI)] which currently awaits CE certification, was further evaluated. **In the second part of results in the current thesis** (Chapter 4)**,** an *in vitro* study was performed by amplifying single cells recovered from the C&R using two strategies for single-cell whole genome amplification. Subsequently the amplification products were analyzed using comparative genomic hybridization (array-CGH) and next-generation sequencing. **The aims of this part of the study are:** 1) to evaluate the potential value of the C&R, which is based on cell enrichment by targeting EpCAM expression similar to the DC01, for isolation and recovery of single cells; 2) to compare two single-cell whole genome amplification methods and optimize for the samples from the current settings; 3) to assess the quality of the captured single cells by downstream molecular analysis. **As a result**, cells captured by the C&R device could be released with efficiencies ranging from 50% to 96%. Detached cells could be recovered at rates of 12% to 50% (recovering efficiency) for downstream analysis. Array-CGH profiles of the recovered single cells shared identical gains and losses compared to genomic DNA from bulk cells from cell culture. Using the Ion Torrent Personal Genome Machine system, several hot spot mutations which were reported frequently can be detected on recovered single cells. Data from both array-CGH and next-generation sequencing analyses indicate that DNA quality of the detached cells was not altered by the C&R procedure.

To conclude, the first part of the thesis demonstrates as a proof-of-principle application via an *in vivo* method to detect minimal residual disease in nonmetastatic high-risk PCa. The second part of the data describe the feasibility of single CTC characterization using an improved device compared to the previous generation. *In vivo* enrichment using DC01 represents a promising potential of enumeration of CTCs with high sensitivity, making it possible to use CTC as a biomarker in early stage of PCa for the prognosis of cancer progression, and to start or modify therapy regimen as

early as possible. Furthermore, the C&R detector was proven to be valuable for single-cell genomic analysis by *in vitro* study.

KURZZUSAMMENFASSUNG

Zirkulierende Tumorzellen sind seltene Zellen, die aus Primärtumoren oder Metastasen stammend in das Blut von Krebspatienten wandern und mit dem Blutstrom zirkulieren. Seit einiger Zeit stehen diese Zellen wegen ihres analytischen Potenzials bezüglich Tumorprogression und Krebstherapie im Fokus der Wissenschaft. Als größte technologische Herausforderung für deren Detektion und Isolation gilt die extrem niedrige Anzahl an zirkulierenden Tumorzellen im Blut von Krebspatienten. Sie ist es, die eine Anwednung in der klinischen Routinediagnostik verhindert. Eine vielversprechende Möglichkeit zur Isolation und Detektion zirkulierender Tumorzellen ist der CellCollector DC01 (DC01, GILUPI GmbH, Potsdam, Deutschland). Diese Isolationstechnik beruht auf einem Draht, der mit Antikörper gegen ein Oberflächenepitop von Endothelzellen (epithelial cell adhesion molecule [EpCAM]) funktionalisiert ist. Der Draht wird über eine Kannüle (Kubitalvene) in den Blutstrom von Patienten eingebracht und erlaubt – als einzige Technologie – eine in vivo Isolation zirkulierender Tumorzellen. Durch die 30-minütige Inkubation des Drahtes im Blut von Patienten ist man nicht mehr auf wenige Milliliter Blut beschränkt, die als Ausgangsmaterial für alle anderen Technologien dienen. Zusätzlich zur Isolation kann nach Anfärbung auch die Anzahl der isolierten Tumorzellen am Draht ermittelt werden.

Bis dato wurden die meisten Untersuchungen zur Analyse von zirkulierenden Tumorzellen an metastasierten Tumorpatienten durchgeführt. Die Aussagekraft als früher prognostischer Marker etwa für die Detektion minimaler Resterkrankungen bei Patienten mit lokalen Primärtumoren ist nicht bekannt.

Kapitel 3 (Teil 1 dieser Arbeit) zeigt den Vergleich zweier unterschiedlicher Methoden – DC01 und CellSearch – zur Enumerierung von zirkulierenden Tumorzellen aus Proben von 51 nicht-metastasierten Hochrisikopatienten mit Prostatakarzinom. Ziele dieser Studie waren, 1) die Evaluierung der Methoden im Vergleich sowie ihre klinische Anwendbarkeit, 2) die Evaluierung zirkulierender Tumorzellen als Marker zur Vorhersage von Rezidiven und Therapieerfolg nach chirurgischer bzw. strahlentherapeutischer Therapie und 3) die Optimierung der Detektion zirkulierender Tumorzellen durch Kombination hochspezifischer und hochsensitiver Methoden geeignet zur Diagnostik im Krebsfrühstadium.

Die Daten zeigen, dass die Anzahl an Patienten mit detektierten zirkulierenden Tumorzellen doppelt so groß war, wenn die Analyse mit dem DC01 statt mit CellSearch durchgeführt wurde (20/51 vs. 9/46 Patienten bzw. 39,2% vs. 19,6%). Darüber hinaus detektierte DC01 vor Therapiebeginn mehr zirkulierende Tumorzellen pro Patient als CellSearch (Spannen: 0 – 15 vs. 0-5; Median = 0 vs. 0; 75-Perzentile: 1 vs. 0). Gepaarte Analysen über alle 86 Patientenproben (vor und nach Strahlentherapie) zeigen, dass DC01 signifikant mehr zirkulierende Zellen detektierte als CellSearch (Wilcoxon-Vorzeichen-Rang-Test, zweiseitig, P = 0.0062). Die statistische Auswertung zeigt jedoch keine Korrelation zwischen erhöhter Anzahl zirkulierender Tumorzellen und klinischen Parametern der Patienten (TNM-Klassifikation, PSA-Wert oder Gleason-Score).

Konsequent weitergedacht in Richtung personalisierte Medizin, reicht die bloße Enumeration zirkulierender Tumorzellen nicht aus. Einmal mit dem DC01 isoliert, können diese zwar ausgezählt aber nicht abgelöst und für weiterführende Einzelzellanalysen genutzt werden. Wir haben daher einen neuen Ansatz zur Isolation mittels Draht mit nachfolgender Ablöse der Zellen entwickelt. Der neue Draht, genannt Catch&Release [CellCollector type Detektor CANCER03 (Catch & Release, C&R), Gilupi GmbH], befindet sich derzeit in der Testphase und ist für klinische Anwendungen noch nicht freigegeben. In Kapitel 4 (Teil 2 dieser Arbeit) wurde eine in vitro Studie zur Evaluation des C&R durchgeführt, in der Zellen nach Isolation vom C&R abgelöst und zwei unterschiedlichen Genom-weiten Amplifikationsmethoden (whole genome amplification, WGA) unterzogen wurden. Die amplifizierte DNA der Einzelzellen wurde mittels Array-CGH (comparative genome hybridization) und Sequenziermethoden der 2. Generation (next-generation sequencing) analysiert. Ziele dieser Studie waren, 1) die Evaluierung des C&R-Drahtes – dessen Isolationsmethode ähnlich des DC01 auf der Interaktion der zirkulierenden Tumorzellen und EpCAM beruht – bezüglich seines Potenzials im Bereich der Einzelzellanalyse, 2) die Evaluierung und Optimierung zweier unterschiedlicher Protokolle zur WGA von Einzelzellen und 3) Untersuchungen zur DNA-Qualität isolierter Zellen mittels molekulargenetischer Analysen.

Die Daten zeigen, dass von allen mittels C&R isolierten Zellen 50% - 96% vom Draht wieder abgelöst warden konnten. Von diesen Zellen konnten 12% - 50% nach Zytozentrifugation für allfällige Analysen (Array-CGH, Sequenzierung) rückgewonnen werden. Die Array-CGH Profile dieser Einzelzellen stimmte überein mit Kopiezahländerungen (copy number changes) der genomischen DNA (isoliert aus der Zellkultur der jeweiligen Zelllinie). Die gängigen Mutationen

der verwendeten Zelllinien wurden in den isolierten Einzelzellen mittels Sequenzierung am Ion Torrent identifiziert. Beide Analysen zeigen, dass die Qualität der DNA einzelner Zellen nicht durch das C&R-Protokoll beeinträchtigt wurde.

Der erste Teil dieser Dissertation zeigt, dass eine Früherkennung von Rezidiven mittels der in vivo Isolations- und Detektionsmethode (DC01) in micht-metastasierten Hochrisikopatienten mit Prostatakarzinom möglich ist.

Der zweite Teil dieser Dissertation beschreibt eine Möglichkeit zur molekulargenetischen Charakterisierung einzelner Zellen unter Verwendung einer neuen Generation von in vivo Detektoren. Die in vivo Anreicherung mittels DC01 besitzt ein hohes Potenziel bezüglich der Enumeration zirkulierender Tumorzellen und erlaubt es, sie als Biomarker im Frühstadium des Prostatakarzinoms, für dessen Progression, sowie als Indikator für frühestmögliche Änderungen in der Therapie einzusetzten. Die in vitro Studien der neuen Generation an Drähten (C&R) hat gezeigt, dass diese für die molekulargenetische Charakterisierung einzelner Tumorzellen geeignet sind.

ABBREVIATIONS

°C	Celsius temperature scale
ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1
ADT	Androgen deprivation therapy
ALK	Anaplastic lymphoma kinase
APC	Adenomatous polyposis coli
AR	Androgen receptor
Array-CGH	Microarray-based comparative genomic hybridization
bp	Basepair
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BSA	Bovine serum albumine
C&R	CellCollector type Detektor CANCER03; Catch & Release
CFSE	Carboxyfluorescein succinimidyl ester
CK	Cytokeratin
CNVs	Copy number variations
CTC(s)	Circulating tumor cell(s)
ctDNA	Circulating tumor DNA
CTM	Circulating tumor microemboli
CTNNB1	Catenin beta 1
DAPI	4',6-diamidino-2-phenylindole
DC01	CellCollector DC01
dCTP	Deoxycytidine triphosphate
DLRS	Derivative log ratio spread
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOP-PCR	Degenerate Oligonucleotide Primed PCR
DTCs	Disseminated tumor cells
EMT	Epithelial–mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
FBXW7	F-box and WD repeat domain containing 7
FDA	Food and Drug Administration
Fig.	Figure

FITC	Fluoresceinisothiocyanat
Ga	Gauge
gDNA	Genomic DNA
HER2	Human epidermal growth factor
HRPC	Hormone refractory prostate cancer
IF	Immunofluorescence
ISET	Isolation by size of epithelial tumor cells
KIT	KIT proto-oncogene receptor tyrosine kinase
LIANTI	Linear amplification via transposon insertion
M	Molar
MALBAC	Multiple annealing and looping-based amplification cycles
Mb	Megabase
mCRPC	Metastatic castration-resistant prostate cancer
MDA	Multiple displacement amplification
neg.	Negative
NGS	Next generation sequencing
OS	Overall survival
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase chain reaction
PD-1	Programmed death 1 protein
PD-L1	Programmed death ligand 1
PFS	Progression-free survival
pos.	Positive
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
QC	Quality control
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT	Room temperature
RT-qPCR	Quantitative reverse transcription PCR
SD	Standard deviation

SEM	Scanning electron microscopy
SMAD4	SMAD family member 4
SMO	Smoothened, frizzled class receptor
SNP	Single nucleotide polymorphism
TP53	Tumor protein p53
T-stage	Tumor stage
WGA	Whole genome amplification

1. INTRODUCTION

1.1. Circulating Tumor Cells and Circulating Tumor Cell Clusters

Circulating tumor cells (CTCs) are cancer cells escaping from the primary cancer or metastasis into peripheral blood. They are thought to be the precursors of cancer metastases ((Pantel et al., 2009, Yu et al., 2011). The presence of CTCs was first observed by a pathologist a century ago. He noticed that some cells in the blood of a patient with metastatic cancer were similar to those in the tumor tissue (Ashworth, 1869). Due to the lack of sensitive detection methods earlier, the critical role of CTCs in the cascades of cancer metastasis hasn´t been researched in-depth until the recent decades. With a Wright-Giemsa stain, Marrinucci *et al.* demonstrated a high degree of pleomorphism in patient CTCs and a similar morphologic spectrum between primary/metastatic tumor cells and CTCs (Marrinucci et al., 2007). Yu *et al.* reported that the epithelial-mesenchymal transition (EMT) which is a crucial developmental program activated during cancer invasion and metastasis, also occurred on CTCs during their translocation from the circulation to remote tissue sites, thus they categorized CTC status into five groups based on expression level of epithelial and mesenchymal markers on CTCs (Yu et al., 2013). Those classifications were correlated to cancer status as well as response to therapies (Yu et al., 2013). The presence of CTCs has been correlated with patient prognosis in metastatic cancers (Cristofanilli et al., 2005, Cohen et al., 2008). Besides, CTC enumeration during cancer treatment is useful for clinicians to the disease status and helps make decisions regarding treatment (Danila et al., 2007, Cohen et al., 2008, Fehm et al., 2010, Punnoose et al., 2012).

1.1.1. Methods for Detection and Enrichment of Circulating Tumor Cells

Generally, the detection of CTCs implies at first enrichment and then identification of these cells. As the presence of CTCs occurs at an extremely low incidence in peripheral blood (Paterlini-Brechot and Benali, 2007), CTC enrichment from peripheral blood has turned out to be a big challenge for years (Alix-Panabières and Pantel, 2014, El-Heliebi et al., 2017). Plentiful assays have been established for CTC enrichment and identification based on biological (membrane protein phenotype) and physical (size, deformability, and electrical charges) properties of CTCs

(**Fig. 1**). CTC identification critically requires high sensitivity and specificity of the platform, in addition CTCs should be isolated as intact cells enable for downstream analysis. Until recently, most CTC detection assays made use of the specific cell surface markers expressed by cancer cells in order to distinguished CTCs from the background of the much more numerous leucocytes. Among all makers, the epithelial cellular adhesion molecule (EpCAM) is used most frequently, as this molecule is often overexpressed on carcinomas which originate from epithelia, and is absent on hematologic cells (Balzar et al., 1999, Went et al., 2004).

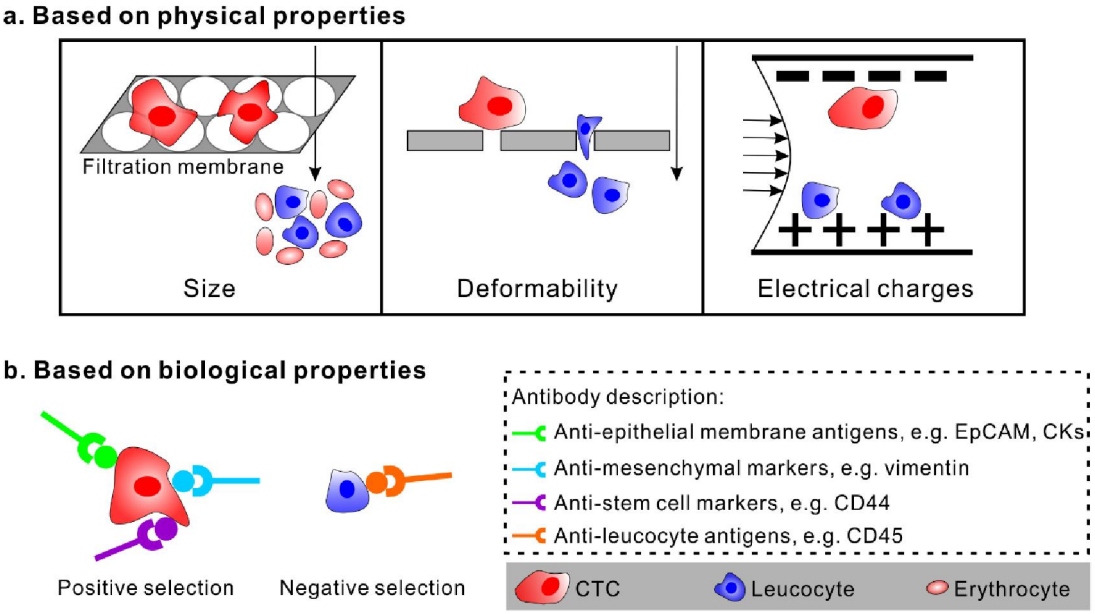

Figure 1. Approaches for CTC detection/isolation [Figure and legend originally published by *Chen S et al.* in *Advances in clinical chemistry, 2017* (Chen et al., 2017b) with minor modifications]**.** (**a**) Physical- property based methods for CTC detection. CTCs (represented by red cells of irregular shape) are generally larger compared to normal hematologic cells (represented by blue cells) and erythrocytes (represented by small red cells of oval shape). Besides difference in size, CTCs are less deformable and show distinct dielectric properties in electric fields. (**b**) Biological-property based methods for CTC detection. CTCs are targeted and enriched by antibodies against epithelial markers (e.g. anti-EpCAM, anti-cytokeratins), stem cell-like markers (e.g. anti-CD44), and mesenchymal markers (e.g. anti-vimentin). Besides, CTCs can be negatively selected using anti-CD45 antibody to deplete CD45 positive leukocytes.

A representative platform is CellSearch system which has been cleared by the U.S. Food and Drug Administration to detect CTCs on metastatic cancers for establishing the prognosis of the disease

(Riethdorf et al., 2007, Sastre et al., 2008, Helo et al., 2009). The availability of the CellSearch detection method was considered a milestone in CTC analysis, and the studies of CTCs on various tumors have been reported since then (Cohen et al., 2008, Miller et al., 2010, Krebs et al., 2011). This semi-automated system requires 7.5 mL of peripheral blood to perform a measurement. CTCs can be targeted and first enriched from other cells in the blood by using anti-EpCAM antibodies bound to magnetic nanoparticles. CTCs are further identified by immunohistochemistry staining with epithelial markers. CK8, CK18 and CK19 are used for positive selection, 4',6-diamidino-2-phenylindole (DAPI) for nuclear DNA staining and CD45 (common leukocyte marker) to identify a possible contamination of leukocytes. The resulting EpCAM+/CK+/DAPI+/CD45- cells are counted as CTCs, and the test result is reported as CTCs per 7.5 mL of blood sample. Due to the minimal invasiveness of this technique, it is beneficial for clinicians to obtain samples repeatedly. Many prospective studies have been performed to validate the specificity, sensitivity and reproducibility of this system to ensure that CellSearch is validated in both scientific and clinical applications (Naoe et al., 2007, Kraan et al., 2011, Nichols et al., 2012). Meanwhile, various comparisons of newly developed methods with CellSearch have frequently been reported in order to compare new techniques to the established gold standard (Van der Auwera et al., 2010, Farace et al., 2011, Andreopoulou et al., 2012, Müller et al., 2012). However, false negative result may occur due to the presence of CTC subpopulations. Some of the CTCs may undergo EMT during traveling in the circulation, as cell surface markers change from the epithelial to the mesenchymal type or co-expressing epithelial, mesenchymal, and stem-cell markers (Armstrong et al., 2011, Kasimir-Bauer et al., 2012, Yu et al., 2013, Joosse and Pantel, 2015)

The CellCollector DC01 (DC01), a medical wire functionalized with anti-EpCAM antibodies (**Fig. 2a**), was first described in 2012 (Saucedo-Zeni et al., 2012) and has been certified as medical device by the EU. Unlike other methods detecting CTCs from volume-limited sample, the detector allows for in vivo isolation and enumeration of CTCs directly from the blood stream. This is done by placing the detector via a cannula into the peripheral vein of cancer patients and EpCAM-positive CTCs are captured (**Fig. 2b**). This type of detector has been CE certified and validated in several cancer studies. In one of a newly published study on lung cancer of varies types (adenocarcinoma, small cell lung cancer and squamous-cell carcinoma), 58% of patients were detected positive for CTCs ($\geqslant$1 CTC) using the DC01, whereas 27% of positive samples were found using the

CellSearch system (Gorges et al., 2015), suggesting that DC01 is more sensitive and efficient in CTC detection.

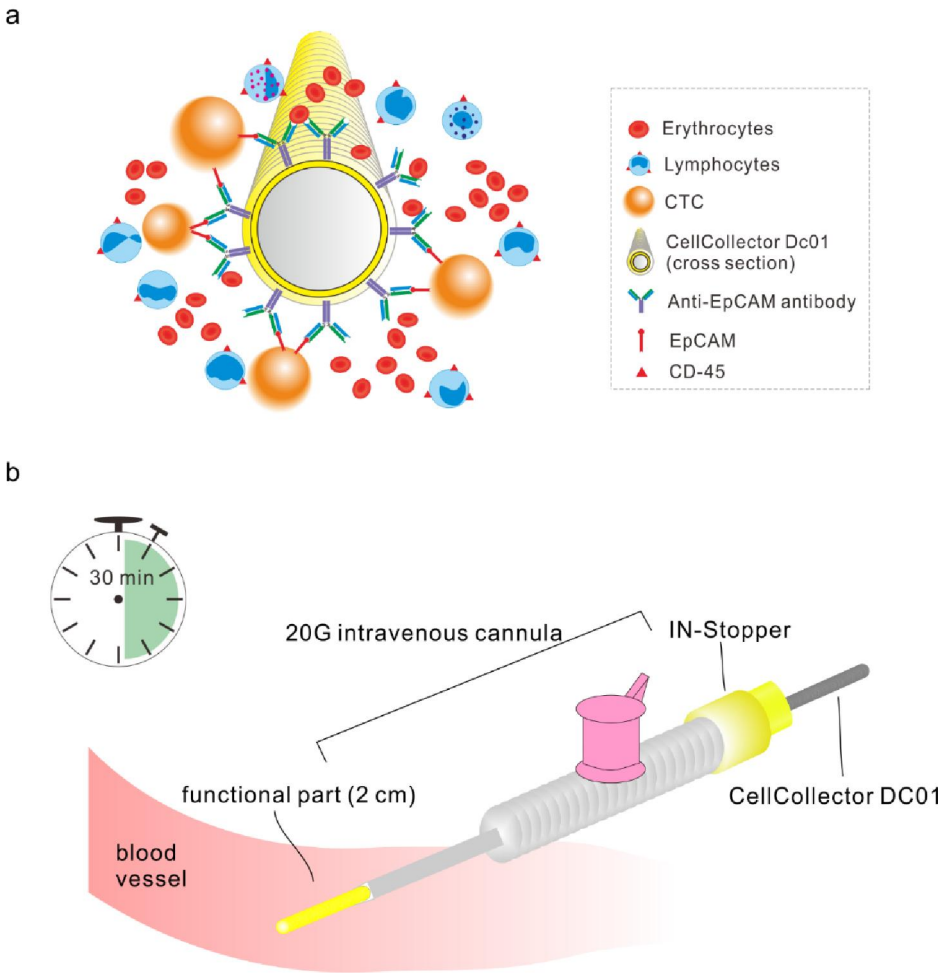

Figure 2. Schematic drawing of CellCollector DC01 and the application procedure (unpublished data; manuscript in preparation). (a) Working principles of DC01 viewed from its cross section. A medical guidewire coated with hydrogel is functionalized with antibodies against Epithelial cell adhesion molecule (EpCAM). Cancer cells expressing EpCAM antigen are targeted and captured onto the 2-cm functional part. (b) Demonstration of application procedure of DC01 in vivo. The CellCollector DC01 is applied into the cubital vein of the patient via a 20-gauge intravenous cannula fixed with a yellow IN-Stopper. The 2-cm functional part is exposed to the blood flow for 30 min allowing the efficient detection of the potential CTCs.

Reverse transcription polymerase chain reaction (RT-PCR) is a sensitive tool which is frequently used to evaluate the expression level of target genes on the level of mRNA. AdnaTest is a platform

combining immunomagnetic beads and RT-PCR to identify and characterize CTCs. The combination of various antibodies that target different subpopulations of tumor cells can greatly increase the sensitivity of downstream assays and help to avoid false-negative results due to factors such as high variability of tumor cell surface antigen expression. Comparisons of CTC detection by AdnaTest and CellSearch have been performed by several studies and diverse results were obtained (Van der Auwera et al., 2010, Andreopoulou et al., 2012, Müller et al., 2012). Combination of both methods may improve the CTC detection rate (Gorges et al., 2016).

CTCs are larger in size compared with other normal blood cells in the circulation, allowing the development of filtration-based approaches (Tan et al., 2009, Mohamed et al., 2009, Lin et al., 2010, El-Heliebi et al., 2013). One representative platform is ISET (Isolation by Size of Epithelial Tumor cells) which is making use of the filter membrane with pores size of 8 µm to isolate CTCs with larger size from blood cells. Isolated CTCs are retained on the membrane for further fixation and labeling by target antibodies. CTC isolation by ISET test is independent on the surface antigen of cells, and the morphology of CTCs can be well maintained for downstream single CTC analysis, which is a simple way and easy to manage. However, CTC size varies a lot and smaller CTCs may be missed during filtration, which is the major limitation of ISET platform. A second EpCAM-independent enrichment method is density gradient centrifugation using Ficoll-Hypaque based on the difference in density between CTCs and granulocytes. OncoQuick is such a platform which has been evaluated in several studies (Gertler et al., 2003, Lagoudianakis et al., 2009, Königsberg et al., 2011). Although the procedure is easy to perform without using any antibody to target cells, this method is of low specificity and somewhat unstable.

A variety of microfluidic platforms based on both physical and biological features of CTCs have been developed for capturing CTCs. The first generation of CTC-chip was covered with 78,000 microposts functionalized with antibodies and etched on a single piece of silicon (Nagrath et al., 2007). The second generation microvortex-generating herringbone-chip (HB-Chip) added some new features based on the performance of the first-generation CTC-Chip. By optimization of increasing blood sample throughput and antigen-antibody interaction, this HB chip increases the ability to capture rare circulating tumor cells by 25% comparing with CTC-Chip (Stott et al., 2010a). CTCs were detected on more than 90% of patients with metastatic prostate cancer (Stott et al., 2010b). In 2013, Ozkumur *et al.* developed the third-generation CTC-iChip (i: inertial focusing)

being capable of isolating both epithelial and nonepithelial cancer cells (Ozkumur et al., 2013). Based on the inertial focusing strategy, leukocytes were first labeled with magnetic beads coated with antibodies. When the blood sample flowed through the microfluidic chamber, the red blood cells, plasma, and remaining magnetic beads were eluted according to the volume (Ozkumur et al., 2013). Labeled leukocytes were further removed in the magnetic field and CTCs were finally obtained in the remaining liquid (Ozkumur et al., 2013). The advantages of this new CTC sorting equipment compared to the previous technologies lie in the successful integration of a number of technologies and expanded ability in processing large volumes of whole blood with high throughput at high efficiency without prior knowledge of the molecular characteristics of these tumor cells (negative selection mode). Moreover, isolating CTCs regardless of tumor surface epitopes allows well-preserved cell viability and makes it possible to culture CTCs *ex vivo* (Yu et al., 2014). Angle's Parsortix CTC capture system is another microfluidic device newly developed based on the deformability and size of CTCs. Using this method, both CK+ and CK- CTCs can be efficiently enriched on one platform and higher number of CTCs can be detected compared to other platforms (Xu et al., 2015, Chudziak et al., 2016, Hvichia et al., 2016, Reduzzi et al., 2017). Recently, this method proved compatible with in situ padlock probe technology, allowing further molecular characterization of captured CTCs (El-Heliebi et al., 2018).

1.1.2. Molecular Characterization of Circulating Tumor Cells

Beyond mere enumeration, characterization of CTCs with respect to their biological properties is of utmost importance to provide important information on (a) cancer metastasis, (b) identification of novel therapeutic targets, (c) mechanism of therapy resistance, (d) stratification of patients for personal treatment, and (e) monitoring of cancer treatment in real-time (Lianidou et al., 2013).

In view of the important role of gene copy number variation (CNV) in cancer development, genetic correlation of CTCs to primary tumor and metastasis is still unclear. Molecular characterization of CTCs may provide crucial information to understand their origin and elucidate the reasons for inter- and intra-patient heterogeneity among CTCs. High-throughput sequencing of the genome-wide and exomes of single peripheral blood CTCs in lung cancer patients revealed reproducible CNV patterns similar to those of metastasis across all CTCs from individual patients with the same cancer subtype and distinct CNV pattern among CTCs from different cancer type (Ni et al., 2013).

In a study on patients with metastatic colorectal cancer, using a panel containing 25 CTC specific genes to perform quantitative reverse transcription PCR (RT-qPCR), Onstenk *et al.* showed that the profile of CTCs is more consistent with the metastatic site (Onstenk et al., 2016). By genomic analysis of CTCs and single cells of primary and metastatic tissue in a colorectal cancer patient, researchers found that the progression of cancer disease is due to the convergent evolution rather than random occurrence (Gao et al., 2017).

RT-qPCR offers high sensitivity to analyze multiple targets in a single tube, which becomes one of the most widely used methods for CTC characterization. In 1991, Smith *et al.* demonstrated for the first time that CTC could be successfully detected by a targeting tyrosinase transcript which encodes a tissue-specific gene in melanocytes (Smith et al., 1991). Since then, this method has been demonstrated to be efficiently combined with other CTC detection platforms to characterize CTCs at the RNA level (Xenidis et al., 2009, Strati et al., 2011, Yokobori et al., 2013). Recently, various multiplex RT-qPCR assays were established to characterize CTCs accurately and efficiently by targeting multiple transcripts in one tube (Sieuwerts et al., 2009, Markou et al., 2018, Strati et al., 2017). The main disadvantage of RT-qPCR in the study of CTCs is that with the enhancement of sensitivity, the false positive rate is also increasing. Thus, a minor contamination of clinical specimens may lead to false positive data. Besides RNA analysis, the feasibility of exploring the methylation status of tumor suppressor genes on CTCs has also been confirmed by methylation specific PCR (Chimonidou et al., 2011, Mastoraki et al., 2015), although clinical relevance needs to be further confirmed by correlation to the outcomes of patients (Mastoraki et al., 2015).

Programmed death-1/programmed death ligand-1 (PD-1/PD-L1) immunotherapy is a new type of anti-cancer immunotherapy, which is making use of the body's own immune system to resist cancer by blocking the PD-1 / PD-L1 signaling pathway. Mazel *et al.* first characterized expression level of PD-L1 in CTCs isolated by CellSearch and expression of PD-L1 was found in 11 of 16 patients with breast cancer (Mazel et al., 2015), which confirmed the feasibility of PD-L1 assessment in CTCs. Recently, Nicolazzo *et al.* evaluated the PD-L1 status in CTCs of non-small-cell lung cancer patients during treatment with the PD-1 inhibitor Nivolumab and short-term follow-up data indicated that the presence of PD-L1(+) CTCs was correlated to disease progression (Strati et al., 2017, Nicolazzo et al., 2016). In another study on locally advanced head and neck squamous cell

carcinoma, patients with an upregulation of PD-L1 mRNA expression in CTCs had unfavorable progression-free survival and overall survival (Strati et al., 2017).

CTC characterization can be also performed by *ex vivo* CTC culture. Epithelial immunospot technology (EPISPOT assay) is a method for short-term CTC culture (Alix-Panabières, 2012). During a period of 48-hour *ex vivo* culture, fluorescence-labeled immunospots representing targeted proteins secreted by viable CTCs can be detected and enumerated. This functional assay has proven useful in characterizing viable CTCs at the single-cell level on patients with different tumor types including breast, prostate, and colorectal cancer (Alix-Panabières et al., 2005, Alix-Panabières et al., 2009, Denève et al., 2013, Ramirez et al., 2014). Because CTCs are derived from solid tumors, *in vitro* culture or *in vivo* models of CTCs may allow preclinical testing of treatment regimens (Maheswaran and Haber, 2015) or individualized patient-acquired drug sensitivity information (Yu et al., 2014). However, there are still many technical problems *in vitro* culture of CTCs yet to be overcome. By separating CTCs for *in vitro* culture, we may be able to monitor the change in drug sensitivity of each patient at different treatment stages. If used in the clinical treatment process, this procedure may help find the most suitable treatment.

1.1.3. Circulating Tumor Cell Clusters

In the peripheral circulation, CTCs may travel in clusters rather than as single cells. CTC clusters are also referred to as circulating tumor microembli (CTM) and they are comprised of at least two cancer cells up to 100 cells. CTC clusters appear to be of higher metastatic potential than single CTCs (Aceto et al., 2014). In a study performed by Aceto *et al.*, a mouse model was used to demonstrate the role of CTM in cancer metastasis (Aceto et al., 2014). Using CTC-Chip technology, they found that the CTM was not a phenomenon of simple cell aggregation in the circulation. Upregulation of plakogolobin (a component of cell desmosomes and skeleton structures) was found in CTC clusters and downregulation of plakogolobin correlated to the dissociation of clusters (Aceto et al., 2014). This study also demonstrated that 35% of patients with late-stage metastatic breast cancer were positive for CTM and higher numbers of CTM were associated with decreased survival rate (Aceto et al., 2014). A significant correlation was also obtained in a cohort of prostate cancer patient. CTC clusters show dynamic changes in expressing epithelial and mesenchymal markers during cancer development, which makes it possible to achieve metastasis efficiently

(Aceto et al., 2015, Fabisiewicz and Grzybowska, 2017). Previously, it was thought that CTC clusters become easily trapped during circulation as they are large in size. However, a study performed by Au *et al.* showed that CTC clusters are deformable to a considerable extent as they pass through narrow capillaries (Au et al., 2016). Overexpression of Ki67 was found within CTC clusters, suggesting that CTC clusters are highly proliferated, which might benefit cancer metastasis and dissemination (Sarioglu et al., 2015). Whether CTC clusters can be used as a prognostic marker in metastatic cancer is still in debate. The formation of clusters as well as the cooperation among cells needs further study (Fabisiewicz and Grzybowska, 2017).

For a specific isolation of CTC clusters, researchers developed a Cluster-Chip technology that captures CTC clusters from untreated blood without relying on tumor-specific markers (Sarioglu et al., 2015). This method separates CTC clusters by specific branch traps and maintains the integrity of the composed cell population under low shear stress. The marker-independent feature makes the Cluster-Chip suitable for capturing CTM from many types of cancers, including those that have lost tumor- characteristic surface proteins during metastasis (Sarioglu et al., 2015).

1.2. Clinical Relevance of Circulating Tumor Cells

CTCs detection and analysis can be widely used in the study on precancerous lesions, malignant tumors, and tumor metastasis. Analysis of the number or genotypes of CTCs and their characteristics will be helpful in many aspects, such as (a) early diagnosis of tumor, (b) monitoring of drug resistance, (c) prognosis and survival analysis, (d) detection of tumor recurrence, (e) evaluation of drug efficacy to assist in treatment decisions, and (f) adjustment of treatment options.

1.2.1. CTC Detection in Metastatic Cancer

The metastatic spread of cancer cells is still a critical reason for cancer-related death (Weigelt et al., 2005). During metastatic procedure, a small group of cancer cells from the primary tumor invades the surrounding tissues, enters the blood system and spreads throughout the body (**Fig. 3**). At distant sites the surviving cells migrate from the blood vessels into tissues where they adapt to the new microenvironment and finally form metastases (Fidler, 2003, Chaffer and Weinberg, 2011). The mechanism of tumor metastasis is still unclear and remains to be verified. Among the tumor cells released into the bloodstream, less than 0.01% of cancer cells are able to survive and

metastasize in peripheral blood (Luzzi et al., 1998). In 2010, a new cM0 (i+) category was added to the cancer staging system to define the status of disseminated tumor cells in circulation or bone marrow without the presence of metastases detected by imaging techniques, which emphasizes the important role of CTCs in metastasis formation (Edge and Compton, 2010, Chen et al., 2017b).

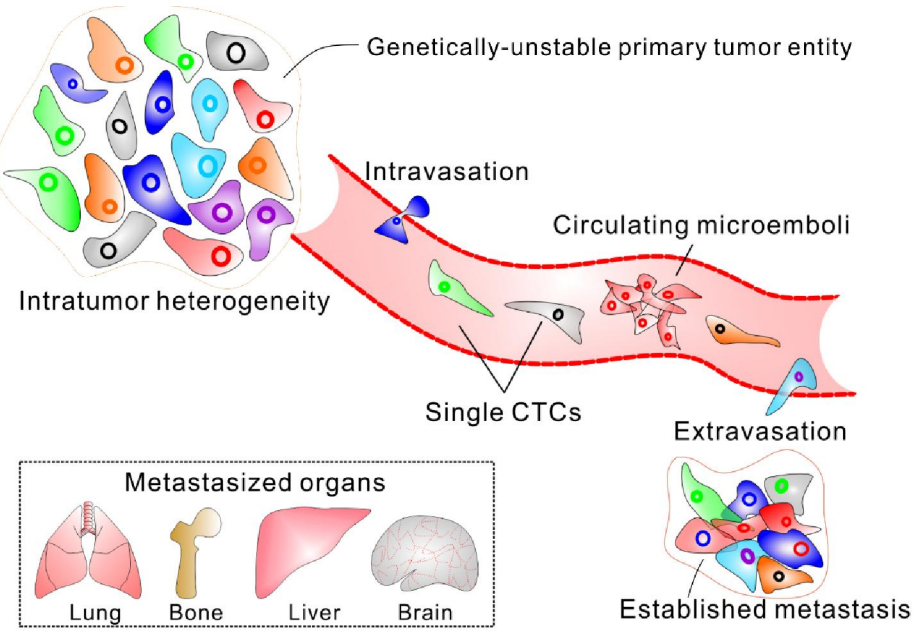

Figure 3. Schematic illustration describes intra-tumor heterogeneity, circulating tumor cells (CTCs)/circulating microemboli (CTM), and their role in cancer metastasis [Figure and legend originally published by *Chen S et al.* in *Advances in clinical chemistry, 2017* (Chen et al., 2017b) with minor modification]**.** Cancer cells with various genetic alterations coexist within a genetically-unstable primary tumor entity (represented by cells of different colors; the same color type of nucleus and cytoplasm). Some of the cancer cells acquire the ability of invasiveness and enter into the circulation followed by traveling to distant organs either as single CTCs or CTM. During the metastatic process, epithelial-originated cancer cells may acquire mesenchymal characteristics, resulting in increased motility (cell alterations represented by distinguishable color types of nucleus and cytoplasm). Finally, CTCs/CTM extravasate to the surrounding tissue and finally establish a distant metastatic lesion. The organs most frequently affected by cancer metastasis are lung, bone, liver and brain as a consequence the organ-specific metastatic colonization of different cancer types.

The value of CTC detection in prediction of prognosis and its evaluation of the efficacy of treatment have been further studied. In 2004, Cristofanilli *et al.* first described the clinical relevance of CTCs in advanced breast cancer. They used the CellSearch system to measure CTCs counts in peripheral

blood from healthy people, patients with benign breast disease, and metastatic breast cancer. The results showed that CTCs were widespread in patients with metastatic breast cancer, but absent in other patient groups. CTCs can be detected in peripheral blood in 70% of patients with advanced breast cancer and ⩾ 5 CTCs / 7.5 mL were found in more than 50% of patients (Cristofanilli et al., 2004). Giordano *et al.* conducted a retrospective study of the relationship between CTC counts and clinical outcomes in 517 patients with recurrent metastatic breast cancer (Giordano et al., 2011). The results showed that patients with higher counts of CTCs before treatment (⩾ 5 / 7.5 mL) had poor prognosis (median survival was 18.3 months vs. 32.4 months, $P < 0.001$). For patients with advanced colorectal cancer, CTC counts before and during chemotherapy and targeted therapy independently predicts disease-free survival and overall survival (Tol et al., 2009).

CTC detection can also be helpful in the development of new therapeutic targets for metastatic cancer, in the study of the mechanism of drug resistance, which benefits decision-making treatment in clinical practice (Pantel and Speicher, 2016, Krebs et al., 2010, Polzer et al., 2014). For example, Carter *et al.* could distinguish chemosensitive disease from chemorefractory disease with high accuracy (83.3% of the cases) by classification of baseline copy-number aberrations (CNAs) identified in CTCs from patients with small lung cancer (Carter et al., 2017). Furthermore, five patients with consistent chemosensitive CNA profiles developed recurrent disease, suggesting that drug resistance has not been acquired during chemotherapy (Carter et al., 2017). In one study on CTC phenotypes in patients with recurrent metastatic breast cancer, patients with CTCs expressing more than two multidrug resistance-related proteins (MRPs) showed worse outcome compared to those with CTCs expressing drug-sensitive proteins (5.4 vs. 19.5 months) (Giordano et al., 2011). Pestrin *et al.* reported that HER2-positive CTCs can be found in the peripheral blood of some patients with HER2-negative breast cancer. Therefore, those patients may also be considered being treated with targeted HER2-targeted drugs such as lapatinib (Pestrin et al., 2012).

1.2.2. Detection of Circulating Tumor Cells in Localized Cancers

Currently, initial diagnosis of cancer depends mainly on conventional imaging methods, established cancer biomarkers and pathological analysis. The limitation of those methods is obvious especially concerning the early stage of the disease. For example, routine imaging can only

detect a tumor of a diameter more than 10 mm. Even making use of the high-resolution PET-CT, the detectable tumor size should be at least 5 mm in diameter (von Schulthess et al., 2006).

Compared to CTC studies performed on metastatic cancers, there are far fewer reports on the correlation between CTCs and early stage cancers. To detect CTCs in early stage cancer proved to be a bigger challenge as CTC counts are extremely rare, thus the threshold has to be set to one CTC to determine CTC positivity as reported in specific studies (Lucci et al., 2012, Zhang et al., 2012, Xenidis et al., 2013). Studies on early-stage breast cancer reported that detection rate of CTCs was variable between 9.4% ~ 48.6% and the presence of CTC(s) was associated with an increased risk of disease recurrence (Apostolaki et al., 2009, Bidard et al., 2009, Lang et al., 2009, Sandri et al., 2010, Riethdorf and Pantel, 2010). The presence of CTCs in early cancer patients without detectable metastasis also suggested that dissemination of cancer cells may initiate prior to cancer progression (Stott et al., 2010b). CTCs can also play an important role in the early diagnosis of some malignant tumors (e.g. pancreatic cancer) with extremely high lethality and rapid progression (Pimienta et al., 2017). Ankeny *et al.* suggested that CTCs were expected to become a diagnostic and staging biomarker for pancreatic cancer (Ankeny et al., 2016). Although the role of CTCs in early stage has been addressed by only few studies so far, the role of CTCs in early-stage cancer will be uncovered for early intervention in cancer disease with the rapid development of CTC detection platforms featured by high sensitivity and accuracy.

1.3. Prostate Cancer and Minimal Residual Disease

Prostate cancer (PCa) is the most prevalent non-cutaneous cancer and the second leading cause of cancer-associated mortality among men in developed countries, with an estimated 164,690 new cases and 29,430 deaths in 2018 in the United States (Siegel et al., 2018). The main risk factors associated with the development of prostate cancer are age, family history, pathological changes, dietary changes, ethnicity, androgen levels, and psychological factors.

Prostate cancer originates from its glandular cells and ducts located in the peripheral zone of the organ. There is no specific symptom in the early phase, and it is a relatively slow growing cancer type, which means that it may take years or even decades before becoming large enough to be detectable. Once the cancer cells spread to other parts of the body and progress to invasive disease,

not only the prognosis is very poor, but also the patients´ quality of life is seriously affected (Donohue et al., 2006, Nelson et al., 2008, Paller and Antonarakis, 2013). Bone is a common metastatic site in PCa, thus, bone metastasis is the main cause of death for late stage of PCa patients (Soloway et al., 1988, Nemeth et al., 2002). More than 80% of the patients that died from PCa presented with bone metastasis (Bubendorf et al., 2000, Keller et al., 2001). During the past few years, the mortality of prostate cancer patients has slightly leveled off by reason of the increasingly wide-spread PCa screening based on prostate specific antigen (PSA) level at an early stage, while this also result in an increased prevalence rate (Schröder et al., 2009, Hugosson et al., 2010, Hoffman, 2011).

PCa is a heterogeneous regarding the molecular nature of tumor entity (Shah et al., 2004, Klein et al., 2014, Boutros et al., 2015). Different subtypes of PCa display a huge variety in DNA level and epigenetic molecular level and its heterogeneous patterns affect the early diagnosis, treatment and prognosis of patients (Perner et al., 2006, Barry et al., 2007, Aryee et al., 2013, Brocks et al., 2014).

1.3.1. PSA Screening

PSA is a glycoprotein enzyme encoded by the KLK3 gene in humans. It is a produced and secreted by epithelial cells of prostate gland. Increased PSA level of more than 4.0 ng per mL are generally considered abnormal. As one of the few reliable markers for cancer screening, PSA test is included as part of the clinical routine for diagnosis of benign and malignant prostate, differential diagnosis, and is an important indicator in the follow-up of PCa patients. However, it is not a tumor-specific marker as elevated PSA level is also associated with other conditions, such as elder age, prostatitis, benign prostatic hyperplasia, or after urinary tract operations. On the other hand, normal PSA level cannot exclude the diagnosis of PCa.

Over recent decades, it has become a concern that PSA-based PCa screening is at the risk of over diagnosis and overtreatment. Several large-scale studies have been conducted in Europe and the United States to determine whether PSA screening reduces the mortality of PCa patients. The European Randomized Study of Screening for Prostate Cancer (ERSPC) showed that PSA screening has reduced cancer deaths by 21% (Schröder et al., 2014), while the United States survey did not observe the same results (Moyer, 2012). In general, the benefit of PSA screening for

prostate cancer remains one of the most controversial topics. Recently, the U.S. Preventive Services Task Force released a guideline about PSA screening (Draft Recommendation Statement: Prostate Cancer: Screening. https://www.uspreventiveservicestaskforce.org/Page/Document/draft-recommendation-statement/prostate-cancer-screening1). In the draft statement, men aged 55 to 69 years were suggested to be well informed by their clinicians and made individual decision about screening for PCa. PSA-based screening was only directly recommended to men aged 70 and older.

1.3.2. Diagnosis and Treatment of High-Risk Prostate Cancer

For the clinical diagnosis, treatment, and prognosis of prostate cancer, three indicators are typically used, i.e. the TNM (tumor-node-metastasis) staging system, the level of PSA, and the Gleason score. Prostate cancers are classified in different ways, one of which is the D'amico Classification System originally developed in 1998 (D'amico et al., 1998). Several variables, such as PSA levels in peripheral blood, Gleason scores, and clinical T-stages, are taken into consideration to categorize patients into low, intermediate, or high-risk of recurrence for the purposes of establishment of prognosis and therapeutic options (D'amico et al., 1998). Among all the newly diagnosed PCa patients, 15% of them are classified in high-risk group (Cooperberg et al., 2010). Patients with high-risk prostate cancer were thought to have a high risk of relapse and mortality from metastatic disease. The survival rate of patients with PCa is closely related to the pathological staging. The 5-year survival rate of localized PCa is nearly 100%. However, as long as it relapses or metastasize to distant organs, the outcome would be very poor with a 5-year survival rate of only 30% (Siegel et al., 2018). Therefore, the early diagnosis of invasive lesions can largely improve the survival rate of patients. Currently the TNM staging describes the disease pathological progression, and the Gleason score is based on tissue morphology. Both of them cannot unravel the genomic alteration during the development of PCa. Moreover, conventional diagnostic techniques such as CT and MRI cannot detect micrometastases. Thus, researchers are trying to find useful prognostic markers for early detection of cancer progression or relapse.

Currently, treatment paradigms for PCa include active surveillance, watchful waiting, radical prostatectomy, radiation therapy, adjuvant androgen deprivation therapy (ADT), cryosurgery, and high-intensity focused ultrasound (Mottet et al., 2017). The main therapeutic goal is the prevention of recurrence and metastasis. At present, the most important treatments for high-risk PCa is still

radical prostatectomy and radiation therapy, but no consensus has yet been reached on the best course of treatment. By evaluating comparative risk-adjusted mortality outcomes, Cooperberg *et al.* demonstrated that radical prostatectomy was more effective than radiation therapy for high-risk prostate cancers and overall survival of the patients was higher (Cooperberg et al., 2010). In 2013, Resnick *et al.* reported the result of a long-term study to evaluate outcomes among men undergoing prostatectomy and radiotherapy (Resnick et al., 2013). After 2 and 5 years of treatment, urinary incontinence and sexual dysfunction were more frequent in patients undergoing surgery than in radiotherapy. In contrast, more complaints about bowel dysfunction were seen in patients following radiotherapy. However, in the long run (after 15 years), there is no much difference in urinary incontinence, sexual function, and intestinal complications (Resnick et al., 2013). In summary, considering the high risk at recurrence, radical prostatectomy combined with extended nodal dissection is considered a reasonable treatment option as suggested by European Association of Urology (Mottet et al., 2017). For patient who prefer radiation therapy, some two to three years of ADT is strongly recommended as an adjuvant therapy by guideline of American Urological Association (Sanda et al., 2017).

1.3.3. Recent Advances of CTC Study in Prostate Cancer

The predictive and prognostic value of CTC enumeration in the setting of metastatic castration-resistant prostate cancer (mCRPC) has been standardized and validated in several clinical trials. For example, a study by De Bono *et al.* evaluated CTC status of 231 patients with castration-resistant prostate cancer (CRPC) using the CellSearch system and also assessed the relationship between overall survival (OS) and pretreatment CTC levels (De Bono et al., 2008). The median overall survival (OS) was 11.5 months in patients with pre-treatment CTCs $\geqslant$5 / 7.5 mL, which was significantly shorter than those with CTCs < 5 / 7.5 mL. Conversion of CTC status before and after chemotherapy affects OS significantly. Similarly, another multicenter study by Miller *et al.* also indicates that patients with unfavorable CTC counts at any time of the treatment (before and after initiation of a new line of therapy) have a poor outcome. Among patients with conversion of CTC status those with persistent CTC counts have the most unfavorable outcome (Miller et al., 2010).

Androgen deprivation agents targeting the androgen-receptor signaling axis is the first-line treatment for advanced prostate cancer. However, the vast majority of patients will eventually develop hormone refractory prostate cancer (HRPC). Thus, it is a critical unmet need to determine the therapeutic efficacy by a more reliable indicator. Moreover, persistent AR transcriptional activity despite AR blockade has been found to mediate resistance to AR-targeted therapy and cancer progression to mCRPC (Dehm et al., 2008, Guo et al., 2009). As a non-invasive approach, analysis of CTCs may predict the therapeutic effect of drugs. Antonarakis *et al.* used AdnaTest technology to separate CTCs and analyzed the expression of AR-V7 using RT-PCR (Antonarakis et al., 2014). Among 31 patients who received abiraterone treatment, the PSA remission rate of AR-V7-positive patients was 0, whereas in AR-V7-negative patients it was 68% (Antonarakis et al., 2014). Prolonged PSA-progression-free survival was seen in the patients with AR-V7 negative compared to AR-V7-positive patients (5.3 vs. 1.3 months). Similar results were also obtained from mCRPC patients treated with enzalutamide (Antonarakis et al., 2014). Furthermore, AR-V7-positive CTCs may be a potential biomarker for selecting treatment (Onstenk et al., 2015, Scher et al., 2016). Recent study on a small cohort of patients with mCRPC and abiraterone/enzalutamide resistance, Gupta *et al.* identified common genomic alterations related to therapy resistance, such as gains in AR and ERG, losses of PTEN and activated proliferative signaling pathways (Gupta et al., 2017).

In PCa, a common recurrent chromosomal aberration is the gene fusion between the androgen-regulated transmembrane protease, serine 2 (TMPRSS2) gene to the ETS family transcription factor ERG, accounting for 40% ~80% of this malignancy (Clark et al., 2007, Hermans et al., 2006). TMPRSS2:ERG gene fusion is a prognostic factor for the recurrence of PCa in surgery-treated patients (Nam et al., 2007). The presence of the TMPRSS2:ERG gene fusion has been correlated to a higher Gleason Score and clinical stage (Demichelis et al., 2007). Based on the significance of the TMPRSS2:ERG gene fusion in the progression of PCa, Attard *et al.* used CellSearch to isolate CTC from patients with castration resistant prostate cancer (CRPC) and further analyzed the relation between ERG gene status and abiraterone acetate treatment. The results showed that ERG gene rearrangement in CTC was homogenous and therapy resulted in PSA decline (Attard et al., 2009). In another study, Danila *et al.* also investigated the expression of TMPRSS2:ERG gene fusion in CTCs. However, their data did not indicate that the presence of the gene fusion gene is

associated with a decline in PSA or a response to chemotherapy of abiraterone acetate (Danila et al., 2011).

After radical prostatectomy of patients with localized PCa, biomedical recurrence was estimated to occur on 15% to 25% of those patients (Pound et al., 1999, Amling et al., 2000, Pal et al., 2015). Conventional diagnostic techniques such as CT and MRI cannot detect micro-metastases of PCa when cancer disease is at an early stage. In consequence, it is quite crucial to diagnose the invasive lesions earlier to improve the survival rate of patients when the cancer is still localized. However, until recently, only a few studies of CTCs at early stage PCa were reported and CTCs are present at much lower concentration in the bloodstream compared to metastatic cancer disease (Stott et al., 2010b, Thalgott et al., 2013, Kolostova et al., 2014, Loh et al., 2014, Thalgott et al., 2015, Meyer et al., 2016), which has brought a great challenge for their study and requires more sensitive, reliable and specific techniques. The presence of CTCs in the peripheral blood of localized PCa patients may be associated to the occurrence of micro-metastasis (Kuske et al., 2016). Some aspects of the study of CTCs in nonmetastatic localized PCa remains controversial, and the number of CTCs is low partly due to limited sensitivity of current detection techniques (Davis et al., 2008, Khurana et al., 2013, Loh et al., 2014). No significant correlation was detected in terms of CTC positivity and tumor T stage, PSA value, and Gleason score (Davis et al., 2008, Meyer et al., 2016). To validate CTC in localized PCa, improved techniques and larger cohorts with extended follow-up period are needed in future.

1.3.4. Prognostic Value of Minimal Residual Disease

Generally, minimal residual disease (MRD) refers to the status of patients who suffered from acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML). It has been found that after treatment when the patient is in remission, there are still minute level of leukemic cells that remain in the patient, although no symptoms or signs of disease can be detected using current diagnostic techniques (van Dongen et al., 1998, Venditti et al., 2000). It was also reported that MRD is the major cause of relapse in leukemia (Coustan-Smith et al., 2004, Basso et al., 2009, Faderl et al., 2010).

Recently, the term MRD was used to describe micrometastatic cells such as CTCs or disseminated cancer cells (DTCs) in solid cancer without any clinical or even histopathological signs of overt metastases (Riethdorf and Pantel, 2010, Müller et al., 2010, Ignatiadis et al., 2011). It is an accurate way to evaluate the treatment response and monitor remission or relapse of cancer disease earlier. CTCs are likely to play an important role in mediating cancer progression and metastasis formation, thus a potential surrogate marker for MRD (Pantel et al., 2008, Kasimir-Bauer et al., 2016). In localized cancer, the detection of CTCs after treatment may indicate the presence of MRD (Köllermann et al., 2008). By molecular characterization of CTCs detected after neoadjuvant chemotherapy on primary breast cancer, Kasimir-Bauer *et al.* described high expression level of a therapy resistance marker (ERCC1: Excision repair cross-complementing rodent repair deficiency, complementation group 1) in CTCs from seven of eight patients treated with taxanes or anthracyclines, suggesting an insufficient initial treatment or development of drug resistance (Kasimir-Bauer et al., 2016).

1.4. Single-Cell Analysis

Heterogeneity of cells is one of the characteristics of organisms. There are huge differences in gene expression among individual cells existing in the seemingly same group of cells. Previously, using cell populations as study objectives obtained only averaged characteristics or only the dominant number of cells prevailed whereas unique characteristics of individual cells are ignored. With in-depth research in cancer, more evidence shows that tumor cell clones in tumor tissue are heterogenous. Advanced tumors are comprised by many tumor cell clones, and each clone has independent characteristics of mutation, morphology, and drug response. Analysis of these single-cell clones can expand our knowledge of cancer cell metastasis and recurrence, and of the mechanisms of therapy resistance.

Single-cell profiling is largely depending on the development of state-of-the-art technologies. Three types of technologies are of utmost importance for single-cell research. First, the most straightforward method is quantitative PCR (qPCR) of which the assays, instrument, and data analysis are relatively simple and well established compared to other sequencing techniques (Ståhlberg and Kubista, 2017). A big advantage of qPCR for single-cell analysis is that all DNA,

RNA and protein from the same single cell can be integrated into one protocol (Ståhlberg et al., 2012).

Second, sequencing technologies has undergone a remarkable progress. Thanks to the appearance of next-generation sequencing (NGS) technologies represented by the Illumina platform, sequencing cost has been greatly reduced and it is possible to sequence multiple samples in one experiment. Third, the development of whole genome amplification techniques makes it possible to amplify single-cell nucleic acid samples and solves the problem of small amounts of initial single-cell nucleic acid materials. The combination of the latter two technologies enables the sequencing of single cell genomics and single cell transcriptomics.

Prior to initiating single cell analysis, target single cells need to be identified and isolated. There are a variety of options for separating single cells from heterogeneous cell populations. In **Table 1**, we compare the performance of applications currently used for single-cell isolation.

Table 1. Comparison of the Most Commonly Used Technologies for Single-Cell Separation

Technology	Sample type	Pros	Cons	References
Micromanipulation	Cell suspension	• Single-cell isolation from heterogeneous cell population • Easily equipped and performed • Unbiased isolation without sample fixation	• Low-throughput, manual • Large volume of initial suspension • Potential inaccuracy	*(Citri et al., 2012, El-Heliebi et al., 2014, Kroneis and El-Heliebi, 2015)*
Laser-capture microdissection (LCM)	Tissue samples	• Investigating heterogeneity of tissue sections • Associating molecular information of individual cells to their specific location in the tissue	• Low-throughput, manual • Pretreatment of tissue (fixation, embedding) • Potential challenge to isolate small cells	*(El-Heliebi et al., 2013, Kummari et al., 2015)*
Fluorescence Activated Cell Sorting (FACS)	Cell suspension	• High-throughput, automatic • Particular sorting using molecular markers • Multi-parametric analyses	• Huge number of cells in starting suspension • Pre-labeling of cells before sorting	*(Schulz et al., 2012, Gross et al., 2015)*
Magnetic-Activated Cell Sorting (MACS)	Cell suspension	• High-throughput, automatic • High specificity and purification	• High running cost • Non-specific contamination from background cells	*(Miltenyi et al., 1990, Welzel et al., 2015)*
Microfluidic	Cell suspension	• High-throughput, automatic • Concentrated template within small volumes • Effective isolation of rare cells	• High cost • High skill needed • Material selection for microfluidic chip	*(Wheeler et al., 2003, Lecault et al., 2012, Mazutis et al., 2013)*
Transcriptome *in vivo* analysis (TIVA)	Tissue samples	• *In vivo* transcriptome analysis using photoactivation • Spatially characterized single cells in live tissue • Noninvasive method for capturing mRNAs in their natural microenvironment	• Low-throughput • Limited number of cells and one type of tissue targeted each time	*(Lovatt et al., 2014)*

1.4.1. Whole Genome Amplification from Single Cell

Generally, one human cell contains only about 6.6 pg of DNA and 10 pg of total RNA. Aiming at obtaining a large amount of accurate data from such a limited size of sample is undoubtedly a huge challenge. Traditional methods of high-throughput analysis in genomics, transcriptome, or proteomics can only be based on a large number of cells. A few years ago, a series of technological breakthroughs have allowed for characterization of transcriptomes and epigenomes for individual cells. Obtaining WGA products of high coverage and sufficient amount is the prerequisite for accurate and comprehensive sequencing results. In order to ensure a high-prevalence and non-preferential amplification of genomes, single-cell WGA technology has undergone several major changes.

The oldest method is Degenerate Oligonucleotide Primed PCR (DOP-PCR) and Multiple Displacement Amplification (MDA). The principle of amplification by DOP-PCR is normal PCR, except that the 3' end of the primer is random (Telenius et al., 1992, Carter et al., 1992). Although the DOP-PCR has some limitations in limited mapping efficiency, amplification bias, and allele dropout (Hu et al., 2016), its performance in detecting copy number variations is reproducible and accurate (Hou et al., 2015). MDA relies on phi29 DNA polymerase featuring exceptional strand displacement activity to achieve isothermal amplification and it is the most prevailing method for WGA (Dean et al., 2002). Based on MDA, Hellani *et al.* first reported successful single cell WGA and identified similar pattern of 16 short tandem repeats between amplified single-cell samples and the genomic DNA (gDNA) (Hellani et al., 2004). The limitations of MDA are biased amplification and high allelic dropout rate, leading to a noisy signal (Hu et al., 2016). In 2012, Zong *et al.* developed the multiple annealing and looping-based amplification cycles (MALBAC) which is a miraculous unicellular genome amplification technology (Zong et al., 2012). This technique is a combination of random linear amplification and tweaked PCR. This WGA method has proven to have higher reproducibility of uniformity in CNVs detection and showed to be more conducive to the heterogeneity research related to variations detection (Zong et al., 2012, Ni et al., 2013, Hou et al., 2015). All of the above techniques are based on random exponential amplification from which the amplification bias and errors cannot be excluded. The emergence of an improved method termed Linear Amplification via Transposon Insertion (LIANTI) technology is a good solution to this problem (Chen et al., 2017a). The entire single-cell WGA is processed by random DNA fragmentation with the Tn5 transposon containing the T7 promoter, allowing linear amplification. This LIANTI method outperforms the existing methods and achieves kilobase resolution to detect micro CNVs. Using this method, the spectrum of single nucleotide changes in single human cells after UV irradiation was determined (Chen et al., 2017a).

1.4.2. Single-Cell Profiling for Precision Medicine

In recent years, some new concepts emerged in the field of medical and health care. Precision medicine was developed with the rapid progress of genome sequencing technology. It is a new medical model based on personalized medicine. With the help of identified new biomarkers and state-of-the-art technologies in genomics and proteomics, precision medicine may accurately find the cause of disease and the target of treatment (Collins and Varmus, 2015). By achieving the

purpose of personalized and accurate treatment of diseases in specific patients, it enhances the effectiveness of diagnosis and treatment of diseases. Multiparameter single cell analysis has unique advantages in promoting the development of precision medicine. First, there is a great heterogeneity in clinical samples. Only single cell analysis can reveal this heterogeneity effectively. Second, many clinical samples are of limited amount, and in extreme case there may be only one cell for analysis. Different from experimental animals, the research methods available to human beings are greatly restricted, so we need to obtain as many data as possible from a limited sample. Third, it is important to diagnose cancer disease precisely based on tumor clones because a minor sub-clonal cell population can be the major cause of tumor proliferation (Marusyk et al., 2014). Tumor heterogeneity is closely related to cancer drug resistance and metastasis (Fidler, 1978, Singh and Settleman, 2010, Turner and Reis-Filho, 2012, Wagenblast et al., 2015). Currently, application of single-cell profiling is already widely used in the field of precision medicine, which is highlightened by a high amount of prospective studies.

Characterizing the instability of genome and transcriptome at the single-cell level we may identify various driver mutations and molecular targets in different cancers (Alix-Panabières and Pantel, 2014, Navin, 2014), which benefits molecular-targeted cancer therapy. Clonal evolution of tumor cells involves a cumulative mutation of the genes that disrupt the normal function of the cell and promote the tumor to transit to a new phenotype (Nik-Zainal et al., 2012, Shah et al., 2012, Landau et al., 2013). Navin *et al.* analyzed copy number variations in two primary breast cancer samples as well as one metastatic sample by single-nucleus sequencing (Navin et al., 2011). Three distinct clonal subpopulations were identified by investigating the clonal structure of one polygenic tumor entity. Interestingly, certain types of cancer cells with diverse genetic alterations were absent in metastasis formation (Navin et al., 2011). In a study on genetic mutations in triple-negative breast cancer, using single-cell genome sequencing technology, researchers identified stable aneuploid rearrangements and point mutations in tumor evolution (Wang et al., 2014). Based on genetic mutation sites, they constructed in addition a phylogenetic tree of clones of tumor cells to further study the mechanism of tumor cloning and evolution, which should be of great help to predict tumor invasion, metastasis, and patient survival (Wang et al., 2014).

In the past, cell lines were frequently used to unravel the mechanism of drug response. However due to the heterogeneity of cells, this did not fully reflect the response of human tissues to drugs.

Single-cell analysis can solve this problem and help us fully evaluate the response of different cells in the tissue to drugs. For example, ADT is a common treatment for PCa, but the efficacy of AR inhibitors after the initial response to castration therapy varies from person to person. Recent studies suggest that constitutively active AR splice variants in exons 5, 6, and 7 can contribute to cancer progression and therapy resistance (Guo et al., 2009, Sun et al., 2010). By single-cell RNA sequencing analysis of CTCs isolated from PCa patients, the noncanonical Wnt signaling pathway (a well-known regulator of cell survival and proliferation) was found to be activated in the therapy resistant group, suggesting the mechanism of drug resistance in PCa (Miyamoto et al., 2015).

DNA methylation has become a biomarker for tumor diagnosis and prognosis (Belinsky, 2004, Brock et al., 2008, Baylin and Jones, 2011). At the single-cell level, genome-wide methylation status is of great significance to reveal the occurrence of cancer, the mechanism of cancer occurrence and development, the heterogeneity of cancer disease, and facilitate early detection, and prognosis evaluation of cancer. Single-cell genome-wide bisulfite sequencing (scBS-Seq) is a single-cell-based WGA combined with a high-throughput sequencing platform to reveal DNA methylation heterogeneity at the single-cell level (Guo et al., 2013, Smallwood et al., 2014, Clark et al., 2018). By performing scBS-Seq on oocytes and mouse embryonic stem cells, Smallwood *et al.* obtained up to 10.1M CpGs (48.4% of all CpGs) at single-cell level and investigated the reproducibility and accuracy of their protocol which should be of great help to further understand the embryonic development mechanism and better treatment of cancer and infertility (Smallwood et al., 2014).

Single-cell analysis has gradually penetrated many related fields of current research. With the continuous improvement and prevalence of relevant technologies, it will continue to coordinate the development of fundamental medical research and clinical practice. In the end, our understanding of our own cells would be the ultimate solution to fight against the disease, since cells constitute the smallest unit of a living organism.

2. MATERIALS AND METHODS

2.1. Patient Recruitment

Informed consent approved by the ethics committee of the Medical University of Graz (25-240 ex 12/13) was obtained from all patients enrolled in this prospective study for the use of their blood specimen. 51 high-risk prostate cancer patients without measurable metastasis were involved according to the D'Amico risk classification (D'amico et al., 1998): PSA $\geqslant$ 20 ng/mL and/or Gleason score $\geqslant$ 8 and/or clinical stage $\geqslant$ T2c staged. Generally, the majority of patients had received a neoadjuvant hormonal therapy for 6-9 months before they started with the radiotherapy. Afterwards then patients were treated with radiation therapy (doses of 78 Gy) with volumetric modulated arc therapy 5 times a week for 7-9 weeks plus long-term concomitant/adjuvant ADT for 2-3 years. Sampling was conducted twice: before the patients started a new line of treatment, and then at the first follow-up visit. Peripheral blood was drawn prior to starting radiotherapy for a baseline evaluation of CTC status and a second CTC evaluation was done over a period of 9 to 19 months after initiation of radiotherapy. For the purpose of comparison, two EpCAM-based methods, CellSearch and DC01, were performed for CTC detection. Two healthy volunteers were subjected to CTC analysis using the DC01 as a control study. The study design and information of sample collection/processing are summarized in **Fig. 4**.

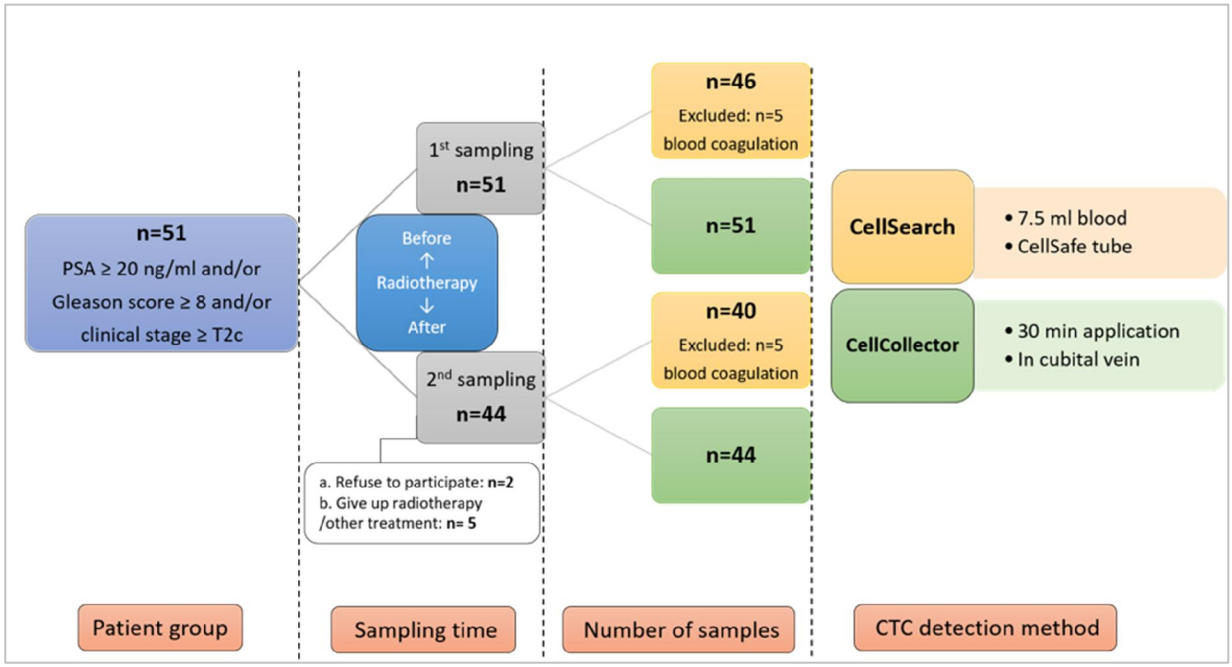

Figure 4. Illustration of study design and patient recruitment (unpublished data; manuscript in preparation)**.**

2.2. Sample Collection and Processing for CTC Detection Using Two Methods

For each patient, the sampling was done twice, i.e. before and after receiving radiotherapy, and three types of blood samples were collected for different CTC analysis at each time as described in **Fig. 3**.

CellSearch: Patient peripheral blood samples were consecutively obtained according to an approved protocol. Blood samples were collected in CellSave Preservative Tubes (Veridex, Raritan, NJ, USA) containing EDTA and cell fixative. Samples were shipped to our collaborating laboratory and processed within 96 h. CTC enumeration using the CellSearch system (Menarini-Silicon Biosystems, Italy) was carried out according to the manufacturer's protocol. Sample preparation was previously described elsewhere (Riethdorf et al., 2007). Briefly, CTC was first magnetically enriched from 7.5 mL of peripheral blood by an anti-EpCAM antibody–conjugated ferrofluid nanoparticles. After washing and permeabilization, immunefluorescent (IF) staining was performed to further identify CTC using CellSearch Epithelial Cell kit (Veridex LLC) which consists of anti-pan-cytokeratin (CK 8, 18, 19) antibodies, anti-CD45 antibodies, and the blue-

fluorescent DAPI nucleic acid stain. A CTC was defined as a nucleated cell (DAPI+) being positive for the expression of pan-cytokeratin and negative for the expression of CD45.

CellCollector DC01: The application of the DC01 has been described previously (Saucedo-Zeni et al., 2012). In short, the DC01 was placed into the cubital vein of the patient via a cannula. After 30 min of application, the detector was collected, washed, acetone fixed and stored at -20°C before further treatment within a period of no longer than one month. For immunofluorescence staining, cells were permeabilized in PBS/0.1% Triton X-100 (Sigmal Aldrich, St. Louis, MO, USA) for 10 min and washed with PBS (pH 7.4). Cells were then blocked in PBS/3% BSA (Sigmal-Aldrich) for 30 min followed by antibody labelling for 1 h. For each sample, 300 µL antibody solution was prepared in 272.25 µL of blocking buffer as follows: Anti-CD45 (MEM-28, AlexaFluor647-conjugated, Exbio, dilution 1:25), anti-pan-cytokeratin [AE1/AE3 (eBiosience) and C11 (Exbio); AlexaFluor488-conjugated, dilution 1:50 (both)], and anti-PSA antibody (H117, AlexaFluor555-conjugated, University of Turku, Finland, dilution 1:80). Hoechst 33342 (Sigmal-Aldrich) was used for nucleus staining with a diluted factor of 1:10, 000 in 1× PBS. The cells were then washed with 1× PBS for 5 min and dried in the air for 5 min.

2.3. Evaluation of CTC Detected by CellCollector DC01

Stained cells were further validated and enumerated by taking images according to the strategies provided by GILUPI Company. Briefly, DC01 was fixed with marked direction on a clear glass slide and positioned on the stage of fluorescent microscope (Observer Z1, Zeiss). The evaluation was carefully performed starting from the bottom end of the functional part to the tip. A CTC was defined as being positive for nucleus staining and pan-CK/ PSA, and negative for CD45. All the CTC images were acquired by the same operator and uploaded to an online image database for independent re-evaluation by our collaborators in order to prevent any evaluation variability.

2.4. Cell Lines

HT-29 cells (a human colon cancer cell line, provided by GILUPI company) were cultured in McCoy's 5A Modified Medium (Life Technologies, Grand Island, NY, USA) supplemented with 2 mM L-glutamine (Life Technologies), 5 mM HEPES buffer, 10% (v/v) fetal bovine serum (FBS,

Gibco), and 1% penicillin- streptomycin (Gibco). LNCaP cell (a prostate cancer cell line kindly provided by Martine Mazel, Laboratory of Rare Circulating Human Cells, Montpellier, France), were cultured in RPMI 1640 Medium (ATCC modification, Gibco) containing 10% FBS and 1% penicillin-streptomycin. PC-3 cells, a human prostate cancer cell lines, originally obtained from the American Type Culture Collection (Rockville, MD), were cultured in Ham's F12 medium (1:1; Gibco) containing 10% FBS and 1% penicillin- streptomycin. All the cells were maintained in T25 cell culture flasks (Nunc) at 37°C under 5% CO_2 atmosphere and were regularly monitored for mycoplasma contamination using a mycoplasma detection kit from Biotool (Houston, TX). For passaging, cells were washed once with 1× PBS (pH 7.4, Gibco) and dissociated by enzymatic treatment using Stempro Accutase solution (Gibco) incubation for 5 min at 37°C. Dissociated cells were washed with culture medium as well as 1× PBS described above and collected into a 15-mL tube for spinning down. The cell pellet was transferred to a new flask for further cell culture. The split ratio was routinely from 1:5 to 1:10. The genetic profiling of each cell line was authenticated by short tandem repeat DNA profiling (Promega).

2.5. Cell Staining and Loading onto CellCollector C&R

For *in vitro* capturing studies using the CellCollector C&R, cells were pre-stained with carboxyfluorescein succinimidyl ester (CFSE, Life Technologies; 5 μM in DMSO) and Hoechst 33342 (Invitrogen, UK) according to the instruction of distributor. Stained cells were rinsed twice in 1× PBS (pH = 7.4) and resuspended in PBS/2% BSA at a density of 100, 000-500, 000 cells per mL. The CellCollector C&R [functionalized with the same anti-EpCAM antibody (humanized HEA125) as the DC01] was incubated with 5 mL of above cell suspension in a 9mL reaction tube (Venosafe) on a horizontal roller mixer (SRT6D, Stuart Scientific) at 5 rpm for 30 min at room temperature (RT).

2.6. Cell Detachment and Single Cell Collection

The detachment step was performed according to the instruction provided by GILUPI. Briefly, the dry powder of GILUPI Release Buffer (GRB) was dissolved by 1× PBS at the final concentration of 4 mg/mL and filtered through a 0.2 μm filter (Corning, NY, USA). The C&R with attached cells was then incubated with pre-warmed GRB solution at 37℃ for 10 min followed by a shake at 200

rpm at RT for 15 min on a plate shaker (DELFIA, Perkin-Elmer). After centrifugation at 300 *g* (Heraeus Megafuge 40R, Thermo Fisher) for 10 min, the C&R was gently removed and GRB containing detached cells was centrifuged again. The supernatant of GRB solution was discarded and collected cells were rinsed twice in 1× PBS, resuspended in 50 µL of PBS/2% BSA, and transferred to 16-Well Lab-Tek Chamber Slides (Nunc, Thermo scientific, Leicestershire, UK). For complete evaluation of the C&R detector and efficiency of GRB, enumeration of cells on the detector was performed before and after cell detachment using GRB and released cells in suspension were counted using a florescent microscope (Axio Observer Z1, ZEISS, Germany). Single cells and cell pools (five to ten cells) were randomly selected using a Zeiss Axiovert 200 inverted microscope as previously reported (El-Heliebi et al., 2014) by a glass capillary (sterile, 20 µm inner diameter, 0° tip angle, Eppendorf, Germany). In short, we transferred 1 µL of 1× PBS containing single cells or cell pools into caps of 200 µL PCR tubes for subsequent WGA using *Ampli1* WGA Kit (*Ampli1*, Silicon Biosystems, Bologna, Italy). For WGA using *GenomePlex* Single-Cell Whole Genome Amplification Kit (*GENOMEPLEX*, Sigma-Aldrich, Dorset, UK), we preloaded the PCR caps with 9 µL of nuclease-free water. We shortly spun down the samples, the cells were immediately manipulated and forwarded to WGA by *Ampli1* or stored at -80°C for later amplification using *GenomePlex*.

We used the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) to extract gDNA from cell line cells according to the description provided by the manufacturer. The amount and quality of extracted DNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Whole Genome Amplification of Single Cells

Amplification using GenomePlex Single Cell Whole Genome Amplification Kit. WGA was performed as previously described with minor modifications (Kroneis and El-Heliebi, 2015, El-Heliebi et al., 2015a). Briefly, single cells were digested using 10 µL fragmentation and lysis buffer, followed by library preparation. Final amplification was performed by adding 7.5 µL of 10× amplification master mix, 48.5 µL of nuclease free water and 5 µL of WGA DNA polymerase.

Amplification using Ampli1 Whole Genome Amplification (WGA) kit. Single cells and cell pools (five to ten cells) were amplified using the *Ampli1* WGA Kit (Silicon Biosystems) according to the manufacturer's protocol version 01 (3-day protocol). Briefly, 2 µL of lysis reaction mix was added to each sample and incubated at 42°C overnight. The digestion reaction mix was added to the same tube the next day and incubated at 37°C for 3 hours. Meanwhile, we prepared the ligation reaction mix by pre-incubating reagents in the thermal cycler. The ligation reaction mix was subsequently incubated with the digested sample at 15°C overnight. Primary PCR Reaction was added to the sample as the last step and DNA was amplified in a multistep reaction in a thermal cycler.

Quality control (QC) of WGA products. For quality control, 1 mL of WGA products were analyzed utilizing a method described previously by EH van Beers (Van Beers et al., 2006). The assays integrated four genomic loci in one multiplex PCR. As positive control, Human gDNA (female/male, Promega Corp., Madison, WI, USA) was used. Amplified products from both WGA and corresponding QC-PCR were visualized and evaluated in 1% agarose gels. Whole genome amplified samples which displayed three to four specific PCR fragments with the sizes of greater than 200 bp in QC assays were expected to result in successful array-CGH profiles. WGA products were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich).

Reamplification of primary linker-adaptor PCR products for array-CGH. To increase the amount of DNA from single cells for variable downstream analysis, a reamplification was performed as described before as an optional step (Geigl and Speicher, 2007, Czyż et al., 2014).

2.8. Array Comparative Genomic Hybridization and Data Analysis

Restriction digestion of reference DNA. Prior to labeling and purification, 250 ng of Human GDNA from a commercial source (Promega, Fitchburg, USA) or reamplified WGA DNA from healthy volunteers (kindly provided by Dr. Bernhard Polzer from Fraunhofer Institute for Toxicology and Experimental Medicine, Regensburg, Germany) was used as a reference. GDNA was subjected to digestion with the restriction enzymes Alu I (R628A, Promega) and Rsa I (R637A, Promega) at 37°C for 2 h followed by inactivation step at 65°C for 20 min. Digested DNA was examined in 1% agarose gel.

Labeling. DNA labeling was done using the BioPrime Total Genomic Labeling System (Life Technologies, Carlsbad, USA) as described previously (Kroneis and El-Heliebi, 2015). Briefly, 250 ng of experimental WGA DNA and 250 ng of reference DNA were labelled with dCTP-Cy5 and dCTP-Cy3 (GE Healthcare, Little Chalfont, UK), respectively. Subsequently, both labelled WGA products and reference DNA samples were purified with Amicon Ultracel-30 filters (UK Millipore, Billerica, USA). Labeling efficiency was determined by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Hybridization. Array-based comparative genomic hybridization (array-CGH) was performed using the SurePrint G3 Human CGH Microarrays 8×60K (Agilent Technologies, Santa Clara, USA). The 8×60K microarrays are constituted of 60, 000 60-mer oligonucleotide probes and the coverage of the human genome is made with a median probe spacing of 41 kb. Experimental and reference DNA were randomly paired, mixed, and denaturation. Pre-annealing was performed at 37°C for 30 min in the presence of Human Cot-1 DNA (15279-011, Life Technologies, Carlsbad, USA). We added the above hybridization mixture on the array slide and hybridized in a rotary oven at 65°C for 24 h at a rotation speed of 20 rpm followed by washing steps according to the manufacturer's instructions, and immediately scanned on a DNA Microarray Scanner (Agilent Technologies).

Data analysis. Feature Extraction software and Agilent Genomic Workbench Lite Edition 7.0.4.0 (Agilent Technologies) were used for processing images and data analysis. Aberrations were examined using the ADM-2 algorithms with a threshold of 8. Centralization Algorithm was set to a threshold of 6.0 with a bin size of 10. To avoid false positive signals and to reduce the background noise, aberration filters were added with the settings defined as follows for both amplification and deletion region: A minimum consecutive probes of three in a given region and minimum absolute average Log2 Ratio of ± 0.25.

Circos plot generation. The aberrations of single cells from LNCaP and PC-3 cell lines were plotted using Circos Tool version 0.21 (Krzywinski et al., 2009). The primary aberration data of average probe log2 ratios were obtained from Agilent Genomic Workbench Lite Edition 7.0.4.0. To generate visualized graph representing copy number variation for samples, a moving average of probe log2 ratio was determined by an arithmetic mean of the series over the past 20 observations based on published TTR package (Ulrich, 2013).

2.9. Comparison of Samples Using Derivative Log2 Ratio Spread Values

In array-CGH, Derivative Log Ratio Spread (DLRS) was generated to show the spread of the intensity log ratio values at regions with similar copy numbers of each sample, which can be used as quality parameter to evaluate the performance quality in the array-CGH experiments of the differently WGA samples. According to the manufacturer's manual the ideal value for gDNA ranges between 0.1–0.2 for an excellent, 0.2-0.3 for a good and > 0.3 for a poor performance which is suggested to be further re-evaluated. It has been reported that for single cell samples, the quoted value is considerably higher, ranging about 1 (Möhlendick et al., 2013).

2.10. Ion Torrent PGM Library Preparation and Next Generation Sequencing

Library preparation. Selected purified *Ampli1* amplified WGA single cell samples were forwarded to targeted next-generation sequencing (NGS). *Ampli1* CHPCustom Beta panel (kindly provided by Silicon Biosystems) was used for DNA amplification in a multiplex PCR. It covers 2265 COSMIC hot spot regions across 315 amplicons of 50 cancer-related genes hotspot regions in 50 cancer-associated genes. Briefly, 10 ng of DNA was amplified using primer panel and 5× Ion Ampliseq HiFi Master Mix. The amplicons were ligated to adapters using the Ion Express Barcode Adaptors Kit (Life Technologies) according to the manufacturer's instructions. After ligation, the library was subjected to a second amplification to complete the linkage. The concentration and quality were determined using the Bioanalyzer High Sensitivity DNA chip (Agilent Technologies). The clonal amplification of the barcoded DNA libraries was carried out using Ion OneTouch 200 kit and subjected to sequencing on a 318 Chip. A quality score of Q20 was used as a measure of successful sequencing.

Data analysis. After the sequencing run, primary data were analyzed using Torrent Suite v.3.4.2. Data were aligned against Human hg19 database using the open source Ion Torrent Suite Software (Thermo Fisher, GPL). Mapping to hg19 and variant calling were performed using the open source Ion Torrent Suite Software (Thermo Fisher, GPL). All called variants were annotated using open source software [ANNOVAR (Wang et al., 2010); SnpEff (Cingolani et al., 2012)] and custom Perl scripts. All coding, nonsynonymous mutations were further evaluated and visually inspected in the Broad's Integrated Genome Viewer (IGV 2.3.66) (Robinson et al., 2011, Thorvaldsdóttir et al.,

2013). Variant calls resulting from technical read errors or sequence effects were excluded from the analysis. The threshold of detected variant frequency was set to 10% mutated alleles among all samples.

2.11. Scanning Electron Microscope Images of Cells Captured by C&R Detector

For SEM observation, C&R detector loaded with HT-29 cells were further treated as previously described (Devetak et al., 2013, Lipovšek et al., 2013). Briefly, the detectors were rinsed in 1× PBS, fixed in 2% formaldehyde, 2.5% glutaraldehyde (w/v) in 0.1 M sodium cacodylate buffer (pH 7.4), and rinsed in the same buffer. Samples were post-fixed in 2% osmium tetroxide solution in the same buffer, rinsed again, dehydrated in a graded series of ethanol (50, 70, 80, 90, and 100 %), and transferred to acetone. Subsequently, samples were dried with liquid CO_2 in a Bal-Tec CPD 030 Critical Point Dryer (Bal-Tec, Los Angeles, California USA), sputter coated under high vacuum with the LEICA EM SCD 500 sputter coating machine (Leica, Vienna, Austria) using gold as a metal target, and visualized using a Zeiss DSM 950 scanning electron microscope.

2.12. Statistical Analysis

The significance between CTC positivity and clinical features was determined using the chi-square (likelihood) test. To compare CTC counts before and after initializing radiotherapy, McNemar's test was used. To compare the efficacy of the two methods (DC01 and CellSearch) for CTC detection the chi-square (likelihood) test was used. Analyses was performed by IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA). Wilcoxon matched-pairs signed rank test (CTC), and Mann-Whitney *U* test were performed using GraphPad Prism version 6.0. All tests were two-sided, and a *P* value of less than 0.05 was considered statistically significant.

3. RESULTS- PART Ⅰ

PART Ⅰ: *In vivo* Detection of Circulating Tumor Cells in Nonmetastatic High-Risk Prostate Cancer Patients Undergoing Radiotherapy

The following data derives from a collaborative project in the frame of ERA-NET on Translational Cancer Research (TRANSCAN) First Joint Transnational Call for Proposals (JTC 2011) on: "Validation of biomarkers for personalized cancer medicine".

Data related manuscript is in preparation as follows:

In vivo Detection of Circulating Tumor Cells in Nonmetastatic High-Risk Prostate Cancer Patients Undergoing Radiotherapy

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Keywords: circulation tumor cells, *in vivo* detection, nonmetastatic prostate cancer, radiotherapy

3.1. Patient Characteristics

The study involved 51 patients with nonmetastatic high-risk prostate cancer between the ages of 55 and 80 years old at entry from November 2013 to April 2017. CTC enumeration was performed before (at baseline; 1st sampling) and after treatment (2nd sampling) using two methods CellSearch and DC01. 44 patients had paired blood samples from baseline and after treatment. All patients received radiotherapy. The median age is 74 years, the median PSA level is 12.0 ng/mL, and the median Gleason score is 8. CTC status was evaluated one day before the start of radiotherapy (baseline statue evaluation) and 44 of them visited for the second sampling within 9-19 months after radiotherapy. Among seven patients who did not visit for the 2nd sampling after radiotherapy, two patients were unwilling to participate the study, three patients gave up radiotherapy, one patient gave up radiotherapy due to the development of bone metastasis, and one patient developed a second cancer and received other treatment.

3.2. Detection of CTCs and CTC clusters using CellCollector DC01 and Correlation Analysis between CTC Positivity at Baseline and Clinicopathologic Features

The short-term application of DC01 *in vivo* for 30 min was well tolerated in all patients without any sign of side effect. Representative images of collector-captured CTC are depicted in **Fig. 5a**, which showed five CTCs detected on three patients. All verified CTCs were positive for the expression of pan-CK (green) and negative for CD45 (red) with intact nucleus (**Fig. 5a**). LNCaP cells were used as positive control for the staining as shown in **Fig. 5b** (upper panel). Additionally, lymphocytes found unspecific binding on the collector were used as negative control for this staining panel as shown in **Fig. 5b** (lower panel). The size of CTC is variable from 4 to 20 μm according to the instruction from GILUPI. Furthermore, in two patients with detectable CTC counts of 10 and 15, respectively, several CTC clusters were identified. Each of the cluster consisted of 2 to 3 CTCs (**Fig. 6**). CTC clusters were not found in other patients with less CTC counts.

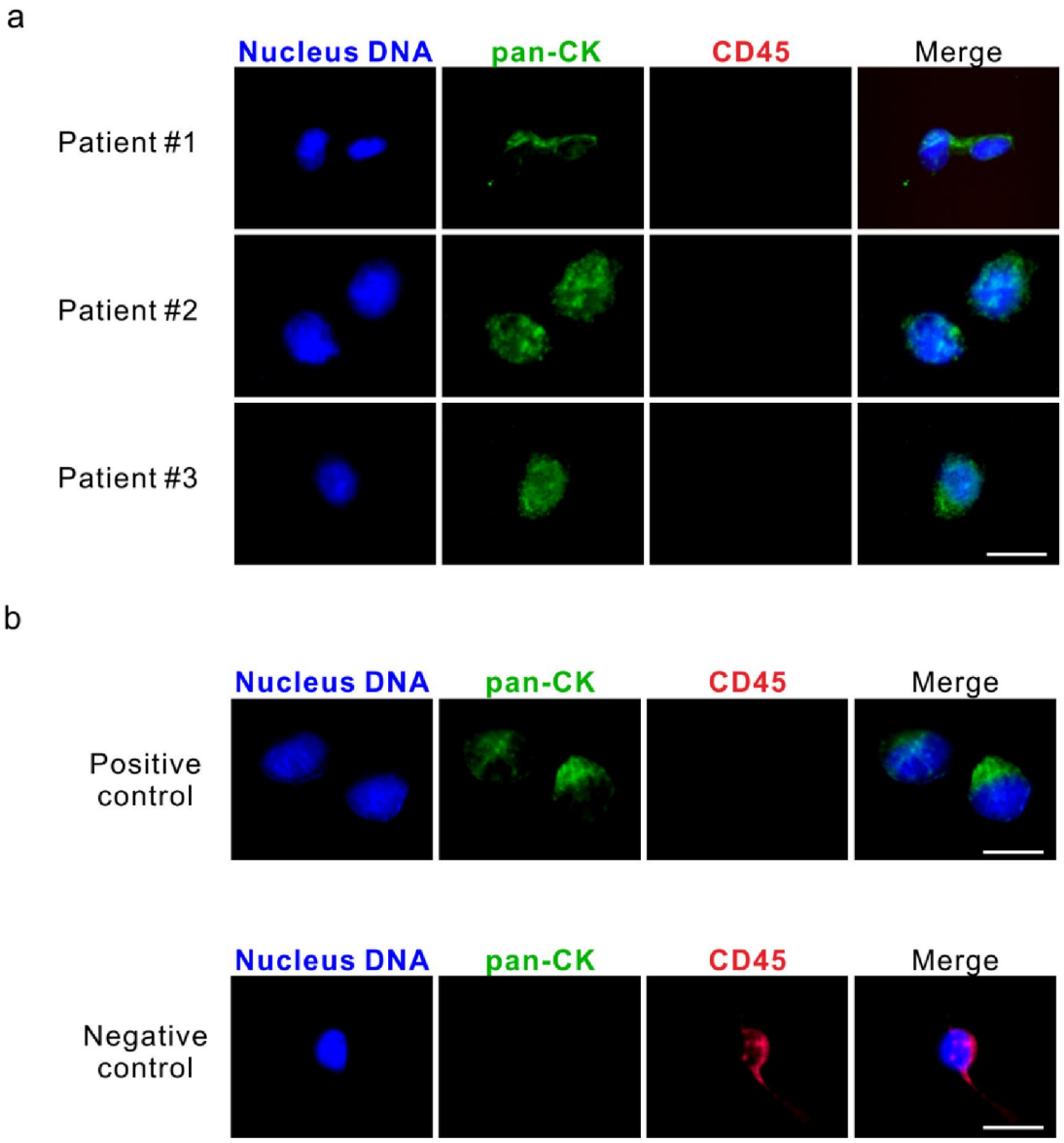

Figure 5. CTCs were detected by CellCollector DC01 in patients with high-risk PCa (unpublished data; manuscript in preparation)**.** (**a**) Micrographs of five CTCs on the DC01 detected from three patients; CTCs were defined as being positive for nucleic staining and pan-CK, and negative for CD45; immunofluorescence staining (DNA (blue) and pan-cytokeratin (green)). All CTCs were negative for PSA, so this channel is not shown. The exposure time for each channel: Nucleus DNA, 800~1000 ms; pan-CK, 3000 ms; CD45, 6000 ms. (**b**) Images of control samples for CTC staining panel, to the upper panel: LNCaP cells (human prostate cancer cell line) captured on the on the DC01 as positive control for CTC staining panel; immunofluorescence staining (DNA (blue) and pan-cytokeratin (green)); to the lower panel: One leukocyte found on the DC01 from one patient sample as negative control for CTC staining panel; immunofluorescence staining (DNA (blue) and CD45 (red)). All scale bars represent 20 μm.

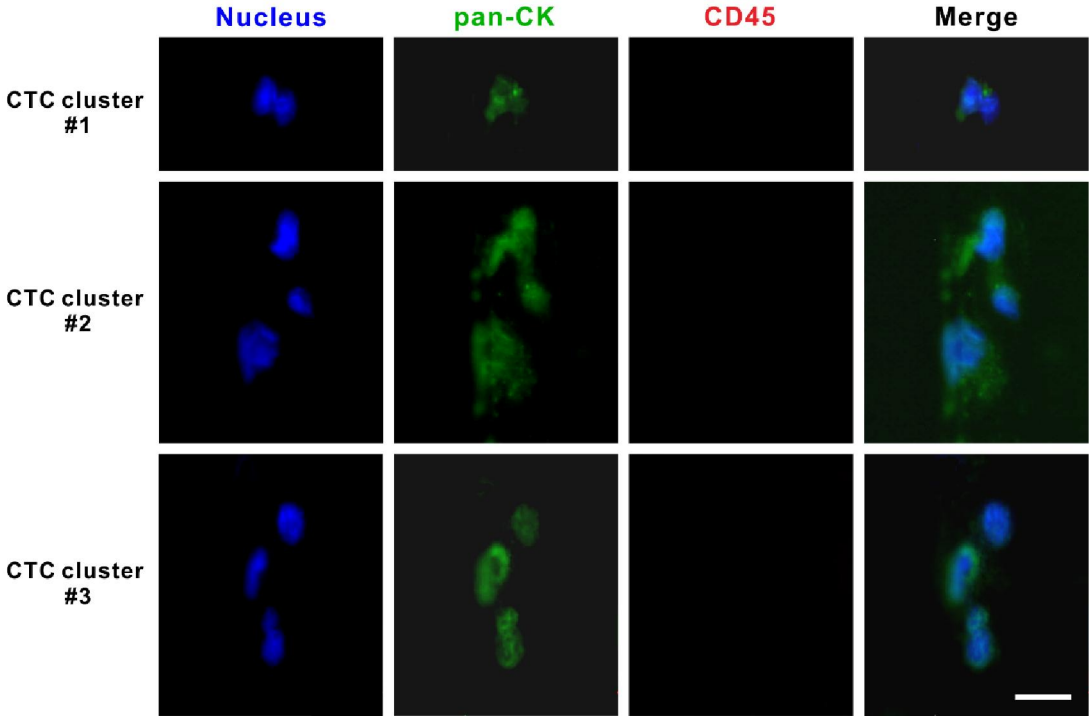

Figure 6. CTC clusters were detected by CellCollector DC01 in patients with high-risk PCa (unpublished data; manuscript in preparation)**.** Three CTC clusters (CTMs) on the CellCollector DC01 detected from two patients. CTMs were found as a cluster of CTCs positive for nucleic staining and pan-CK, and negative for CD45; immunofluorescence staining (DNA (blue) and pan-cytokeratin (green)). All CTCs were negative for PSA, so this channel is not shown. The exposure time for each channel: Nucleus DNA, 800~1000 ms; pan-CK, 3000 ms; CD45, 6000 ms. Scale bar represents 20 μm.

Enumeration of CTCs at baseline was successful in all 51 patients using DC01. As in localized cancer disease, CTCs were quite rare and in positive patients their number was very low, so the cut-off for defining sample as positive sample was set to one CTC, which was also in accordance with previously published data (Davis et al., 2008, Loh et al., 2014, Thalgott et al., 2015, Gorges et al., 2015, Kuske et al., 2016). Detection of CTC by the DC01 revealed that 39.2% (20/51) of patients were positive (**Table 2**). At baseline, three patients presented with greater than or equal to 10 CTC event. The clinical features were collected, including T stage, Gleason score, baseline PSA level, and stratified by CTC status (**Table 3**). Statistical analysis revealed that there was no correlation between CTC positivity and clinical characteristics of patients (**Table 3**). We further summarized the clinical data of patients with detectable CTC using DC01. Patients with detectable

CTCs did not show a worse cancer status (T stage, Gleason score and PSA) than patients without (**Table 4**). Mann-Whitney *U test* showed that there was no significant difference of clinicopathological factors (T-stage, PSA value, Gleason score) between CTC-positive and CTC-negative patient groups. Three patients diagnosed with very high risk of cancer were detected as CTC negative by both methods during two sampling time points (**Table 5**). Importantly, applying DC01 in two healthy individuals did not show capture of EpCAM-positive cells similar to previous report (Gorges et al., 2016).

Table 2. CTC status detected by two methods before and after radiotherapy (unpublished data; manuscript in preparation)

	CTC Positivity (⩾1 CTC)	***CellSearch***	***CellCollector DC01***
1st visit (n=51)	% of positive samples (positive # / total #, %)	**20** (9/**46,** 19.6)	**39** (20/**51,** 39.2)
	range	0-3	0-15
	75th percentiles	0	1
	median	0	0
2nd visit (n=44)	% of positive samples (positive # / total #, %)	18 (7/**40,** 17.5)	27 (12/**44,** 27.3)
	range	0-5	0-14
	75th percentiles	0	1
	median	0	0

Table 3. Summary of clinical characteristics of patients[a] stratified by CTC status at baseline based on two CTC detection methods (unpublished data; manuscript in preparation)

Parameter	Category	CellSearch				CellCollector DC01			
		Total (n=46)	CTC status		*P* value	Total (n=51)	CTC status		*P* value
			positive (n=9)	negative (n=37)			positive (n=20)	negative (n=31)	
T-stage	T1	21	7	14		24	10	14	
	T2	14	1	13	0.097	15	3	12	0.121
	T3	11	1	10		12	7	5	
PSA value (ng/ml)	<10	18	4	14		19	5	14	
	10-20	15	3	12	0.424	16	8	8	0.896
	>20	13	2	11		16	7	9	
Gleason score	6	1	0	1		1	0	1	
	7	2	0	2	0.508	2	1	1	0.579
	8-10	43	9	34		48	19	29	

[a] High-risk prostate cancer patients were included according to D'Amico Risk Classification: prostate specific antigen (PSA) > 20 ng/mL and/or Gleason score ⩾ 8 and/or clinical stage ⩾ T3.

Table 4. Selected features of CTC-positive patients grouped by CTC counts detected by CellCollector DC01 at baseline (unpublished data; manuscript in preparation)

CTCs counts	Total # of patients with specified CTC count	Clinical features in detail		
		Clinical T-stage	PSA value (ng/mL)	Gleason score
1	12	1	10.70	8 (4+4)
		1c	51.44	8 (4+4)
		1c	21.90	7 (3+4)
		1c	14.01	10 (5+5)
		2	11.21	9 (4+5)
		2	27.70	8 (4+4)
		2-3	12.90	8 (4+4)
		3	2.90	8 (4+4)
		3	18.73	8 (4+4)
		3	10.50	8 (4+4)
		3b	9.00	8 (4+4)
		3b	65.00	8 (4+4)
2	4	1c	6.22	8 (4+4)
		1c	5.62	10 (5+5)
		1c	34.60	8 (4+4)
		1c	17.70	8 (4+4)
4	1	2	12.00	8 (4+4)
5	1	2b	4.92	8 (4+4)
10	1	1c	52.10	8 (4+4)
15	2	1c	10.12	9 (4+5)
		3	30.20	8 (4+4)

Table 5. Patients with high risk of cancer status and negative for CTC detected by both methods (unpublished data; manuscript in preparation)

Patient code	T-stage	PSA level (ng/mL)	Gleason score	Pre-therapy CTC count		Post-treatment CTC count	
				CellSearch	DC01	CellSearch	DC01
PGS034	2	**104.9**	**9**	0	0	0	0
PGS038	**3**	**65.72**	**9**	0	0	0	0
PGS044	**3b**	7.37	**10**	0	0	0	0

3.3. Analysis of Paired Blood Samples Before and After Radiotherapy Using Two Methods

We analysed CTC counts of paired samples before and after radiotherapy analysed in parallel by CellSearch and the DC01. We did not find a significant decline of CTC positivity after treatment (**Fig. 7**, upper panel) (McNemar`s test: $P = 0.424$), in contrast to data published earlier (Kuske et al., 2016). A decline was also not observed with the CellSearch system ($P = 1.000$, **Fig. 7**, lower panel).

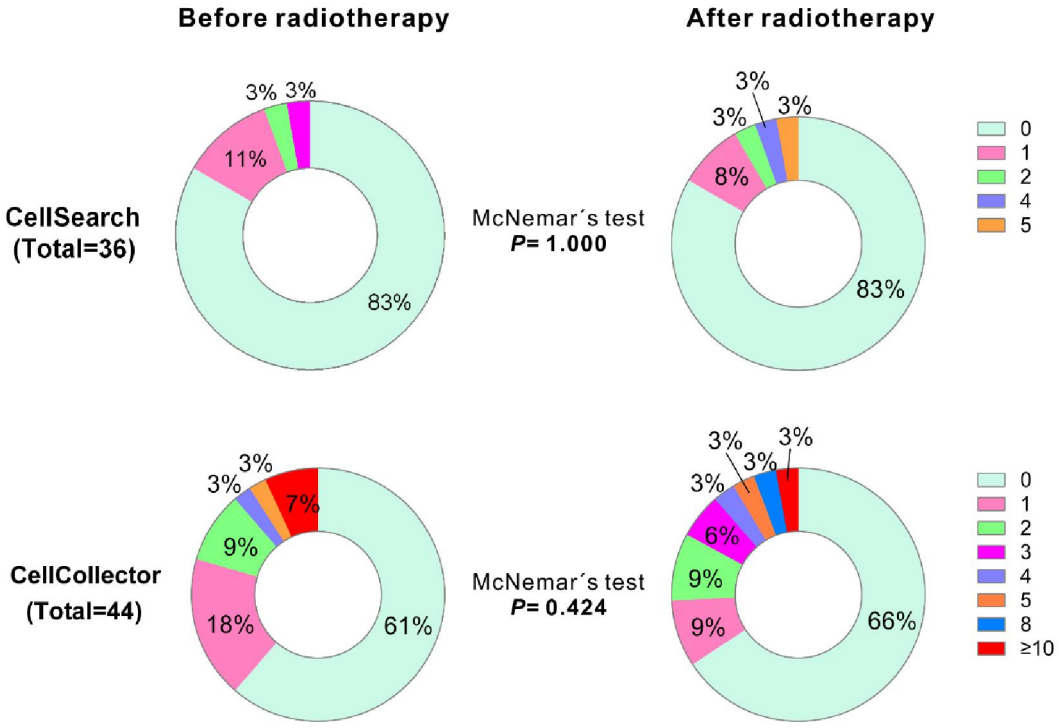

Figure 7. Distribution of CTC counts detected by CellSearch and CellCollector DC01 at two sampling time points (i.e. one day before starting radiotherapy and a few months after receiving treatment; unpublished data; manuscript in preparation; unpublished data; manuscript in preparation)**.** For statistical analysis the McNemar's test was performed ($P < 0.05$ statistically significant, two-tailed).

3.4. CellCollector DC01 Improves CTC Enrichment Efficiency in High-Risk Prostate Cancer Patients

Before treatment, we assessed the base-line CTC status of 51 patients by comparing detection rates of CTC positive samples by CellCollector DC01 to that by CellSearch system. Although both methods are based on EpCAM-enrichment and cytokeratin staining, before initiation of treatment DC01 reported almost twice as many patients to be CTC-positive (39%, 20 of 51) as compared to CellSearch (20%, 9 of 46) with higher numbers of CTCs per patient detected by DC01 (range = 0-15; median = 0; 75th percentile = 1) than by CellSearch (range 0-3; median = 0; 75th percentile = 0) as shown in **Table 2**. After treatment, 44 patients visited for the second sampling. Among them, four samples could not be analyzed by CellSearch due to blood coagulation (n = 4). We identified

that CTC were present in 7 of 40 (18%) patients using CellSearch (range = 0-5; median = 0; 75th percentile = 0), and 12 of 44 (27%) patients using DC01 (range = 0-15; median = 0; 75th percentile = 1).

This difference was found to be significant in all of 86 samples at both sampling time points (**Table 6** and **Fig. 8a**). The percentage of CTC positivity detected by DC01 (33.7%) is significantly higher than by CellSearch (33.7% vs. 18.6%, McNemar´s test, $P = 0.024$). Furthermore, higher numbers of CTCs were detected by DC01 than that by CellSearch as shown in **Fig. 8b**. The plot links the number of CTCs detected by means of parallel enumeration using DC01 and CellSearch. DC01 detected significantly higher numbers of CTCs than CellSearch (Wilcoxon matched-pairs signed rank test, two tailed, $P = 0.0062$).

Table 6. CTC counts of all samples (before and after radiotherapy) explored by two methods (unpublished data; manuscript in preparation)

			CellCollector		Total
			negative	*positive*	
CellSearch	*negative*	Count	49	21	70
		% using CellSearch	70.0	30.0	100.0
		% using DC01	86.0	72.4	81.4
	positive	Count	8	8	16
		% using CellSearch	50.0	50.0	100.0
		% using DC01	14.0	27.6	18.6
Total		Count	57	29	86
		% using CellSearch	66.3	33.7	100.0
		% using DC01	100.0	100.0	100.0

a

	% of CTC positivity (n=86)	*P* value (McNemar test)
CellSearch	18.6	0.024
CellCollector DC01	33.7	

b

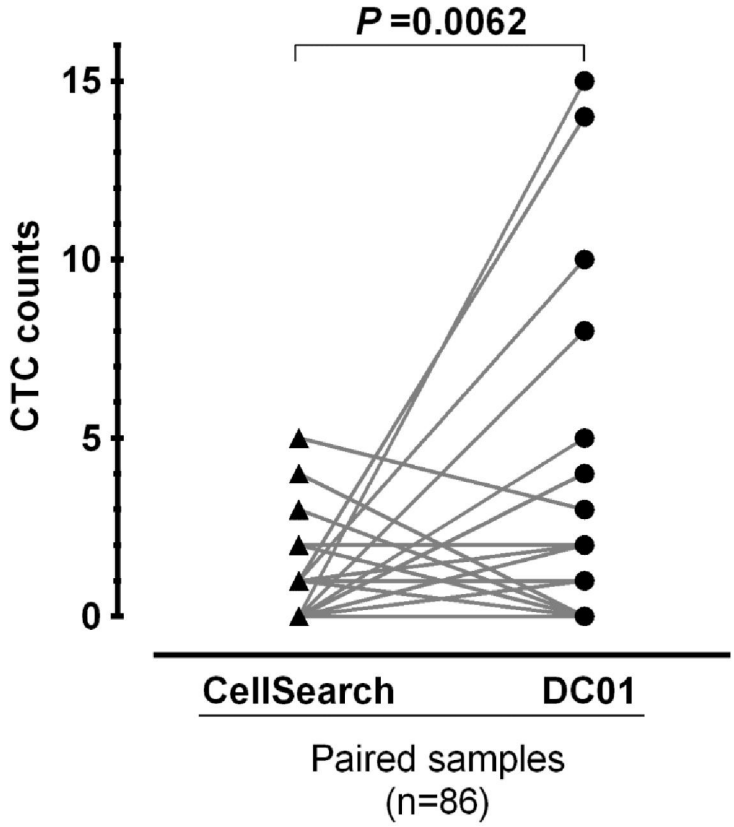

Figure 8. Comparison of CTC positivity and CTC counts examined by two CTC detection methods (unpublished data; manuscript in preparation)**.** (**a**) Comparison of CTC positivity of both methods. For statistical analysis the McNemar's test was performed ($P < 0.05$ statistically significant). (**b**) Comparison of CTC enumeration by parallel analysis of patient samples using CellCollector DC01 and CellSearch (Wilcoxon matched-pairs signed rank test, two tailed, $P = 0.0062$).

In a cohort of 86 patient samples, concordant results were obtained in 8 samples (9.3%) which were positive for CTCs, while 49/86 (57.0%) of samples had no CTC detected in both assays (**Fig. 9a**). CTC counts obtained from both methods were not correlated (r = 0.141, *P* = 0.125, Spearman correlation coefficient) (**Fig. 9b**).

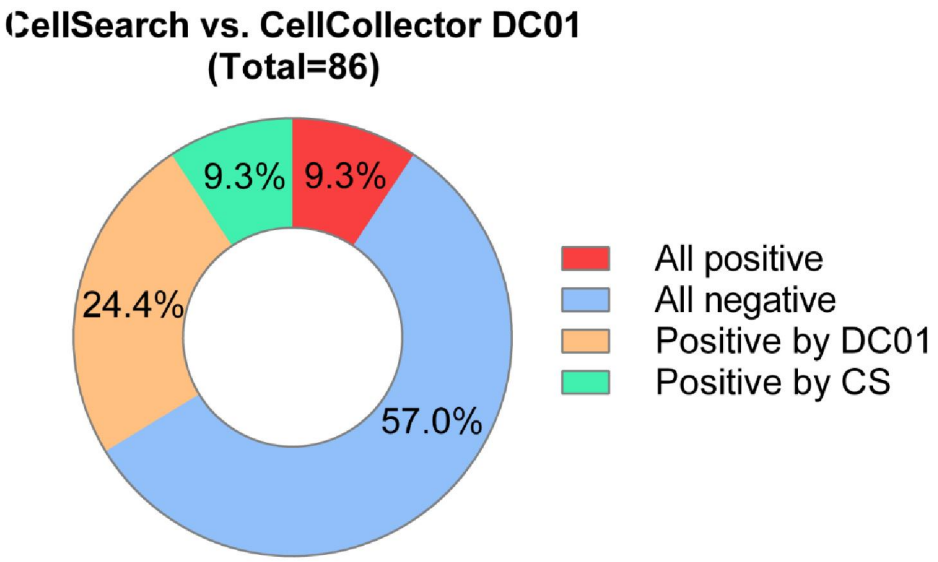

Figure 9. Concordance between two CTC detection methods (unpublished data; manuscript in preparation). Assays were not concordant if CTC status of one sample was different detected by both methods (aquamarine and navajowhite), meaning one assay was positive and the other negative. Assays were supposed to be concordant when the CTC status was the same for one sample detected by both techniques (positive: orangered or negative: <lightskyblue>).

3.5. Conversions of CTC Status

Among all 51 patients available for DC01 analysis, 44 had paired blood samples taken before (baseline) and after radiotherapy. Of the 27 patients negative for CTC at baseline, 17 (63.0%) remained CTC negative (**Table 7**), and the remainder converted to being positive for CTC (n = 10, 37.0%). Of the 17 men with baseline CTC positive status, two (11.8%) remained being positive for CTC, and the remainder converted to CTC negative (n = 15, 88.2%). Among 46 patients available

for CellSearch analysis, 36 had paired blood samples from baseline and post-radiotherapy. Of the 30 patients negative for CTC at baseline, 24 (80.0%) remained CTC negative (**Table 7**), and the remainder converted to being positive for CTC (n = 6, 20.0%). All of the six men with baseline CTC positive status converted to CTC negative (n = 6, 100.0%).

Table 7. Summary of CTC status detected by both methods before and after radiotherapy in paired blood samples (unpublished data; manuscript in preparation)

Detection method	# of patients of different CTC status during sampling period (%)			
	consistently neg.	neg.→pos.	consistently pos.	pos.→neg.
DC01 (n = 44)	17 (39)	10 (23)	2 (4)	15 (34)
CellSearch (n=36)	24 (67)	6 (17)	0	6 (17)

4. RESULTS- PART Ⅱ

PART Ⅱ: *In vitro* Evaluation of A New Type of CellCollector for CTC Detection and Downstream Single Cell Analysis

This part of data has been published as follows:

Chen, S; El-Heliebi, A; Tauber, G; Langsenlehner, T; Pötscher, M; Kashofer, K; Czyż, ZT; Polzer, B; Riethdorf, S; Kuske, A; Leitinger, G; Pantel, K; Kroneis, T; Sedlmayr, P. **Catch and Release: Rare cell analysis from a functionalised medical wire.** *Sci Rep*. 2017; 7: 43424. Published online 2017 Feb 24. doi:10.1038/srep43424

4.1. Highly Efficient Cell Capture Using a CellCollector with Modified Structure and Effective Cell Detachment by Enzymatic Digestion

Although CellCollector DC01 showed promising application in high-risk prostate cancer, the detected CTCs were firmly binding to its surface which prevent the downstream molecular analysis of those cells. In contrast, using a modified structure of the functional part and increased length, the C&R detector allowed more CTCs to be captured and subsequently released by enzymatic treatment (**Table 8**). The functional part of the C&R detector consisting of three strings with a helix structure (**Fig. 10a**). This structure may be helpful to introduce turbulence when applying the device in the blood stream, leading to a higher chance in capturing of EpCAM positive cells. Similar to the CellCollector DC01, on each string of the functional part anti-EpCAM antibodies are chemically functionalized on a polymer layer (**Fig.10b**). After the EpCAM positive cells are captured by the antibodies (**Fig.10b**, upper panel), the detector is incubated with GRB for the purpose of releasing the cells. This enzymatic buffer acts on the polymer to dissolve it and so to release the cells (**Fig.10b**, lower panel).

Table 8. Comparison of features between CellCollector DC01 and C&R detector [table originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]

Type	**Features of Functional part**			**Cell detachment**	**Clinical application**
	Length	*Antibody-conjugation material*	*Structure*		
DC01	2 cm	Hydrogel	Single wire, flat surface	No	Yes (CE certified)
C&R	4 cm	Polymer	Triple helix	Yes	No (*in vitro* use only)

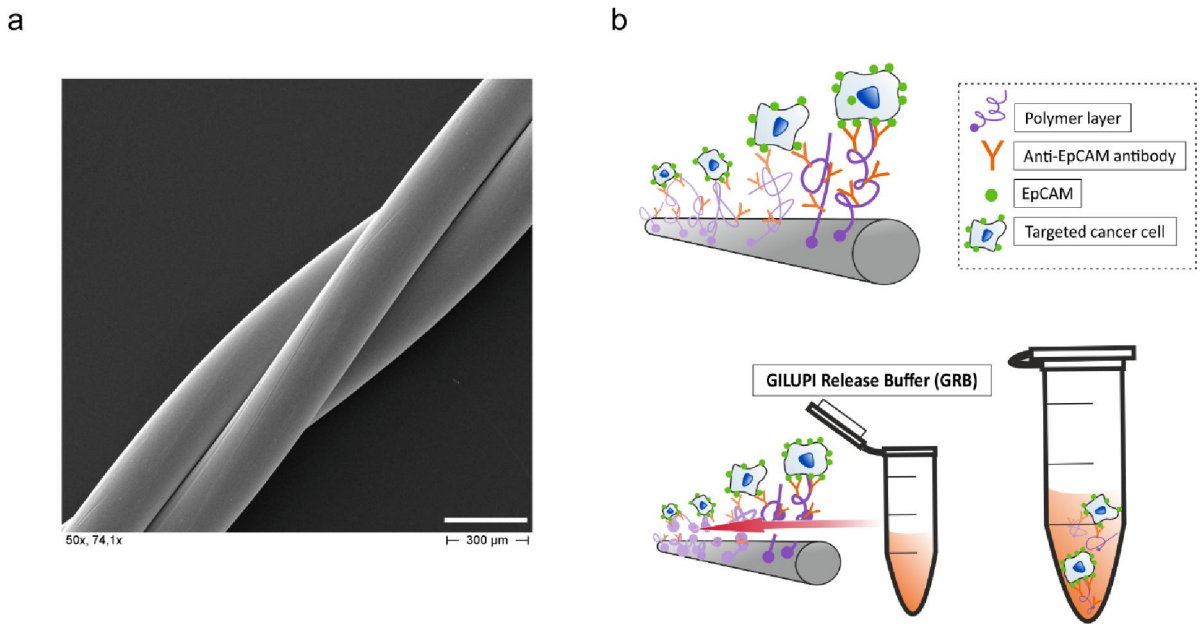

Figure 10. Scanning electron microscope image of the C&R detector and schematic drawing of its polymer layer between functionalized detector and anti-EpCAM antibodies [Part of this figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]**.** (**a**) Scanning electron microscope image shows a three-string structure of the C&R detector. Scale bar: 300 µm. (**b**) The C&R detector is coated with anti-EpCAM antibodies for targeting positive cells during 30 min of incubation. Next, C&R detector with targeted cells was treated with GRB, which acts on the polymer layer to release the captured cells into suspension for downstream single cell collection and analysis.

Figure 11a demonstrates the procedure of *in vitro* evaluation of C&R detector. Pre-stained cultured cancer cells are shown in **Fig. 11b**. They are incubated with the detector on a flat roller mixer (**Fig. 11c**). The detachment procedure is performed in a 1.5-mL LoBind microcentrifuge tube and the C&R detector was fixed using a rubber stopper (**Fig. 11d**). Released cells are re-suspended into cell suspension and ready for micromanipulation for single cell samples. In **Fig. 11e**, one cell was targeted (left panel, red arrow) and manipulated into a capillary (right panel, red arrow).

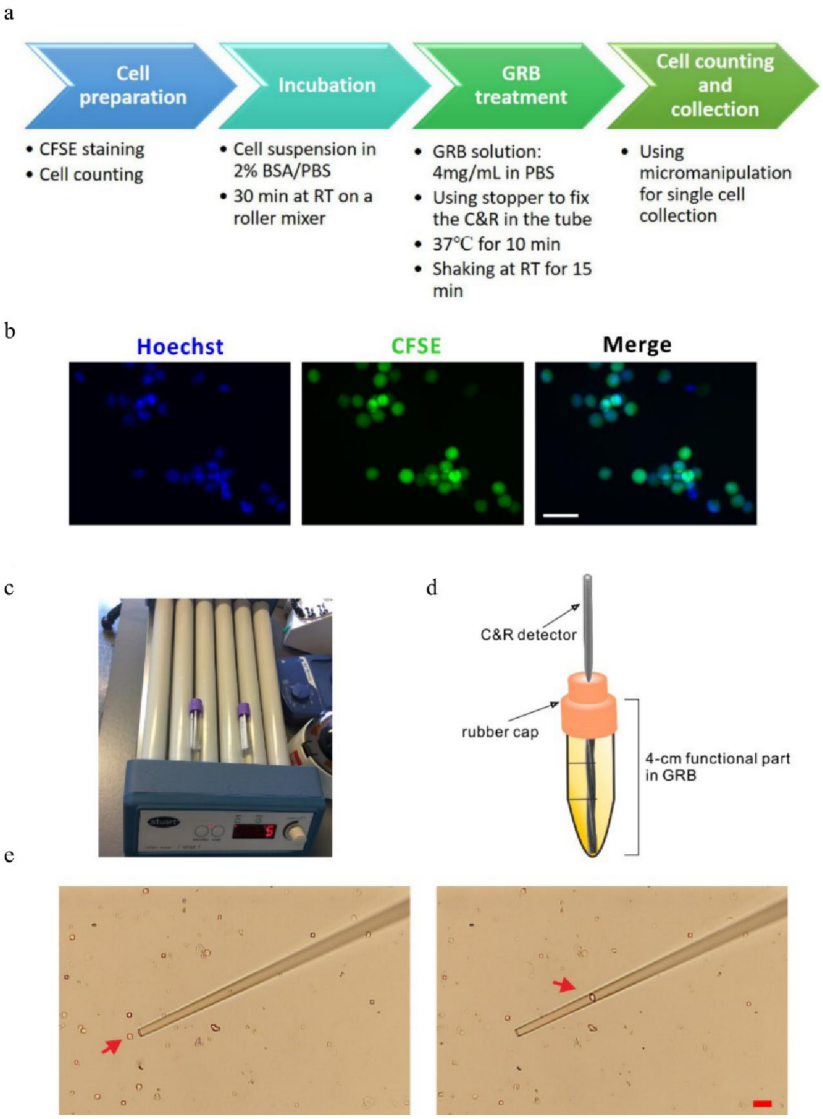

Figure 11. Description of work flow using CellCollector Catch and Release (C&R) for the *in vitro* study. (**a**) Procedure of cell treatment for evaluation of C&R detector. (**b**) Immunofluorescent images of stained cells by the division tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE). HT-29 cells (human colon carcinoma cell line) were pre-stained by CFSE (green) and Hoechst 33342 (blue) for cell tracking and counting before and after C&R treatment. Scale bar: 20 µm. (**c**) Incubation of pre-stained cells (suspended in 2% BSA/PBS at specific density) with C&R detector in a EDTA tube on a flat roller mixer at a speed of 5 rpm for 30 min. (**d**) Schematic drawing of GRB (GILUPI release buffer) treatment to detach cells on the C&R detector. The detachment procedure was performed in a 1.5-mL LoBind microcentrifuge tube. The C&R detector was fixed using a rubber stopper. (**e**) Single cell collection by micromanipulation. Cells released from the C&R detector were re-suspended in 1× PBS. Cell suspension was transferred onto a chamber slide and single cell (indicated by red arrows) was selected into a glass capillary (ΦA=20 µm). Scale bar: 20 µm.

After incubating cells with the C&R detector, SEM images of detector showed that most captured cells are located in the groove of the helix of the detector and bind firmly (**Fig. 12a**). The C&R detector is coated with a polymer layer susceptible to enzymatic treatment (**Fig. 10b**). Accordingly, captured pre-stained cells (**Fig. 12b**) can be released from the detector and collected for single-cell molecular analysis. After detachment of cells from the detector by GRB, cells re-suspended on a chamber slide showed good morphology and viability as seen by CFSE staining (**Fig. 12c**).

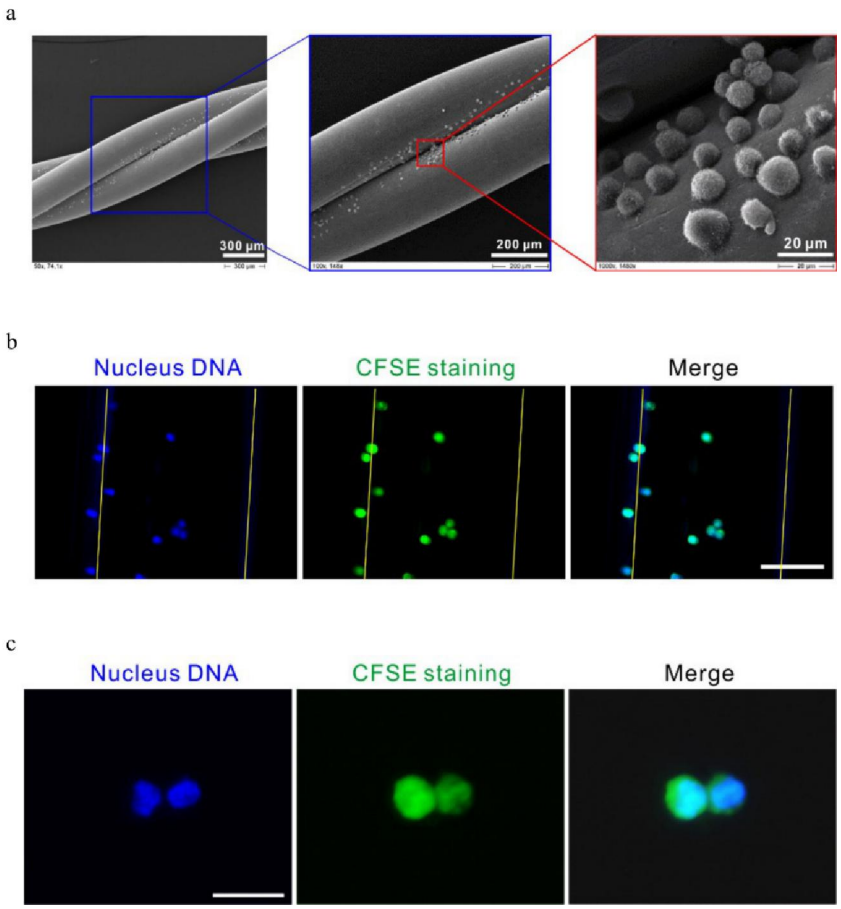

Figure 12. Targeted cells captured by C&R detector [Figure and legend originally published by *Chen S et al.* in *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]**.** (**a**) Scanning electron microscopic images of a C&R detector with binding cells. Left: C&R detector made by three strings forming helix structure (scale bar: 300 µm); middle: captured HT-29 cells in the groove of C&R (scale bar: 200 µm); right: captured HT-29 cells viewed with high magnification (scale bar: 20 µm). (**b**) Immunofluorescence image of the captured HT-29 cells on C&R stained with Hoechst 33342 (blue) and CFSE (green); the parallel yellow lines in each of the images indicate the border line of C&R detector; Scale bar: 20 µm. (**c**) Immunofluorescence image of two detached HT-29 cells stained by Hoechst 33342 (blue) and CFSE (green); Scale bar: 20 µm. Images were taken when cells were suspended in 1× PBS on a multi-well chamber slide.

[The following part of result originally published by *Chen S et al.* in *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]

We investigated the C&R detector's capacity of isolating and detaching pre-stained cells by spiking 1×10^2, 1×10^3, and 1×10^4 HT-29 cells per mL in 5 mL of peripheral blood (n = 3 for all). 30 minutes of exposure to the wire resulted in between 7 and 113 isolated cells across all applied spiking protocols (**Table 9**). When forwarded to enzymatic treatment re-evaluation of the slides yielded average detachment efficiencies of 52%, 62%, and 74% (**Fig. 13a**). Control spiking experiments of 1×10^2, 1×10^3, and 1×10^4 HT-29 cells per mL in PBS/2% BSA (n = 2 for all) resulted in average detachment rates of 65%, 75%, and 72%, respectively. Exposure of the C&R to 5×10^5 HT-29 (n = 5) and LNCaP cells (n = 6) in PBS/2% BSA resulted in between 117 and 393 and between 74 and 754 isolated cells, respectively. The detachment of cells performed similar in both cell lines with mean efficiencies of 81.3% (range between 54.9% and 93.2%) and 81.1% (range between 63.51% and 92.87%), respectively (**Fig. 13**).

Table 9. Enumeration of cells on the CellCollector C&R and in the final suspense during "Catch and Release" procedure [Table originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]

DETECTOR #	CELL LINE	# OF SPIKE-IN CELL PER ML	SPIKING BACKGROUND	CELL COUNTS ON C&R (BEFORE DETACHMENT)	CELL COUNTS ON C&R (AFTER DETACHMENT)	# OF RELEASED CELLS	DETACHMENT EFFICIENCY (%)
1	HT-29	100	EDTA blood	88	36	52	59.1
2	HT-29	100	EDTA blood	54	30	24	44.4
3	HT-29	100	EDTA blood	82	39	43	52.4
4	HT-29	100	2% BSA/PBS	24	9	15	62.5
5	HT-29	100	2% BSA/PBS	32	11	21	65.6
6	HT-29	1,000	EDTA blood	31	11	20	64.5
7	HT-29	1,000	EDTA blood	15	8	7	46.7
8	HT-29	1,000	EDTA blood	28	7	21	75.0
9	HT-29	1,000	2% BSA/PBS	39	6	33	84.6
10	HT-29	1,000	2% BSA/PBS	71	24	47	66.2
11	HT-29	10,000	EDTA blood	113	45	68	60.2
12	HT-29	10,000	EDTA blood	22	5	17	77.3
13	HT-29	10,000	EDTA blood	7	1	6	85.7
14	HT-29	10,000	2% BSA/PBS	20	8	12	60.0
15	HT-29	10,000	2% BSA/PBS	72	12	60	83.3
16	HT-29	100,000	2% BSA/PBS	117	8	109	93.2
17	HT-29	100,000	2% BSA/PBS	206	93	113	54.9
18	HT-29	100,000	2% BSA/PBS	300	70	230	76.7
19	HT-29	100,000	2% BSA/PBS	256	23	233	91.0
20	HT-29	100,000	2% BSA/PBS	393	36	357	90.8
21	LNCaP	100,000	2% BSA/PBS	74	27	47	63.5
22	LNCaP	100,000	2% BSA/PBS	142	34	108	76.1
23	LNCaP	100,000	2% BSA/PBS	505	36	469	92.9
24	LNCaP	100,000	2% BSA/PBS	724	91	633	87.4
25	LNCaP	100,000	2% BSA/PBS	754	80	674	89.4
26	LNCaP	100,000	2% BSA/PBS	79	18	61	77.2

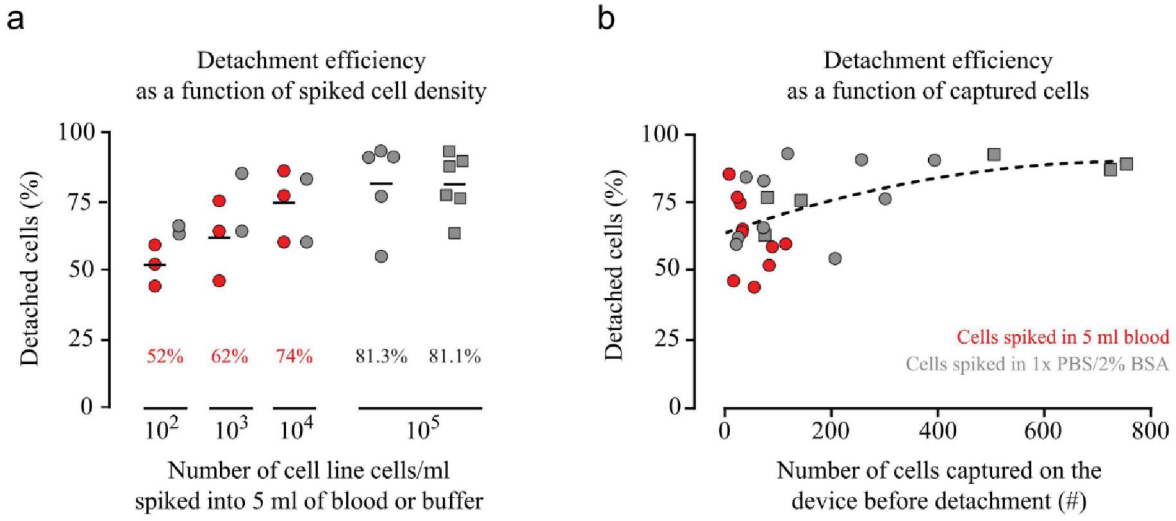

Figure 13. Evaluation of CellCollector C&R *in vitro* [Figure and legend originally generated by Thomas Kroneis and published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c)]**.** Detachment efficiencies are shown as a function of density of spiked cell (**a**) and (**b**) number of captured cells before detachment, respectively. Horizontal bars indicate mean detachment efficiencies. (**b**) HT-29 (represented by circles) and LNCaP (represented by squares) cells were spiked into 2% BSA/PBS buffer (grey) or blood (red) at various densities and incubated with CellCollector C&R for 30 min. Cell enumeration was performed before and after GRB treatment. Dashed line is to guide eye only.

4.2. Single Cell Micromanipulation and Whole-Genome Amplification of Single Cells from C&R detector

We were interested in optimizing WGA methods for single cells collection in our settings and investigating if the C&R procedures have an impact on the DNA quality of single cells. We randomly selected recovered single cells and cell pools (containing 5 cells each) from cell suspension after being released by GRB onto a chamber slide using standard micromanipulation methods (Geigl and Speicher, 2007). To obtain high-quality WGA product suitable for single cell downstream analysis, the amplification of single-cell DNA was accomplished by two different methods for whole genome amplification technologies, i.e. *Ampli1* and *GenomePlex*, and the efficiency of both methods was compared on multiple levels. Gel-electrophoresis analysis of WGA

products from QC-PCR analysis (El-Heliebi et al., 2015b) showed that a higher number of single cells processed with *Ampli1* displayed three more bands of correct size than single cells processed with *GenomePlex* (9/12 vs 6/12 cells, **Fig. 14**). The WGA products showed a smear ranging from 100 to 1,500 bp with a mean size of 400 bp (**Fig. 14**).

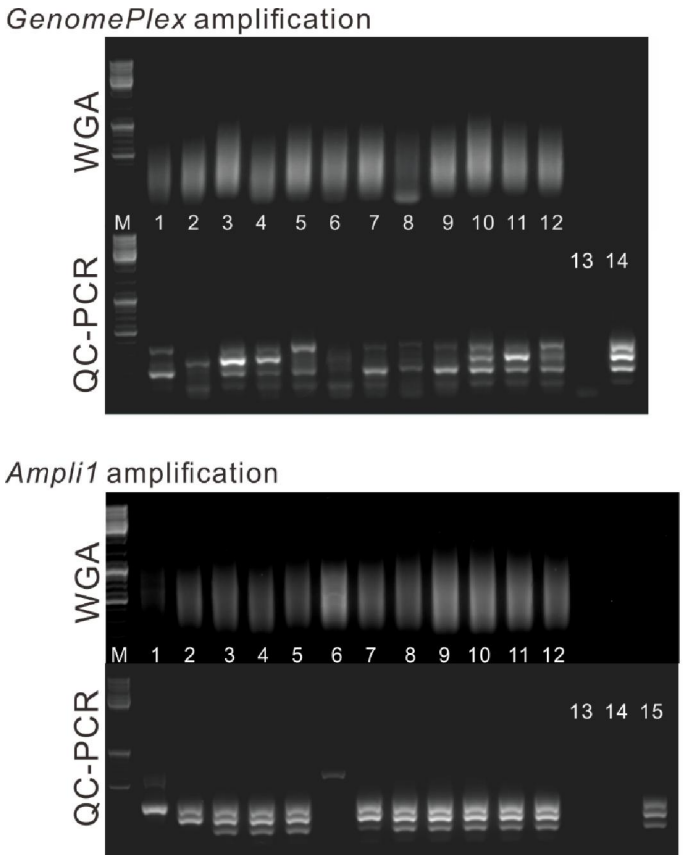

Figure 14. Agarose gel analysis of cell samples of different sources amplified by *GenomePlex* and *Ampli1* and corresponding PCR products of quality control [Part of figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]. WGA products from *GenomePlex* or *Ampli1* were forwarded to QC-PCR to assess the performance of the both WGA methods. PCR products of this QC-PCR as well as aliquots of their corresponding original WGA products were analyzed on agarose gels. Samples with specific range of WGA smears [range 300 - > 1000 base pairs (bp); maximum at about 300-500 bp] which show at least three bands with correct size in QC-PCR products are considered to be of good quality. Upper panel shows lanes 1-8: single cells; lanes 9-12: cell pools (n = 5); lane 13: negative control; lane 14: positive control. Samples in lanes 3-5 and lanes10-12 showed ⩾ 3 bands. Lower panel: lanes 1-8: single cells; lanes 9-12: cell pools (n = 5); lanes 13 and14: negative control; lane 15: positive control. Sample in the lanes 3-5 and lanes 7-12 showed ⩾ 3 bands.

The Derivative Log Ratio Spread (DLRS), which is a widely used parameter to measure the quality of array-CGH experiments, was statistically analyzed for each sample to evaluate the performance of both WGA methods and sampling procedures (**Table 10**). WGA products generated by *Ampli1* harboured lower DLRS values than *GenomePlex*, in general less than 1 (**Fig. 15,** indicated by dashed line), this way within the threshold recommended for single cell samples based on previous study ((Möhlendick et al., 2013). We did not find a difference comparing single cells of HT-29 cells or of LNCaP origin cells when they were amplified by *Ampli1* [DLRS mean values 0.66 (n = 6) vs. 0.63 (n =5), P = 0.6231; **Fig. 15a**]. Meanwhile, comparable mean DLRS values were observed between single cells directly isolated from cell suspension and those detached from C&R [DLRS mean values 0.60 (n = 5) vs. 0.71 (n = 6), P = 0.0923; **Fig. 15b**]. Importantly, regardless of collection resources, single cells amplified by *Ampli1* showed good DNA quality with lower values than *GenomePlex* amplified samples [DLRS mean values 0.65 (n = 11) vs. 1.39 (n = 5), $P < 0.0001$; **Fig. 15c**].

Table 10. DLRS values of single cells from two tested cell lines amplified by two methods

Amplification method	Cell line		Amplification method	Single cell collection (HT29)	
	HT29 (**n=6**)	LNCaP (**n=5**)		Suspension (**n=2**)	C&R (**n=3**)
	0.65	0.8		0.84	1.61
	0.86	0.56		1.86	1.1
Ampli 1	0.66	0.62	*GENOMEPLEX*		1.55
	0.54	0.6			
	0.71	0.57			
	0.56				

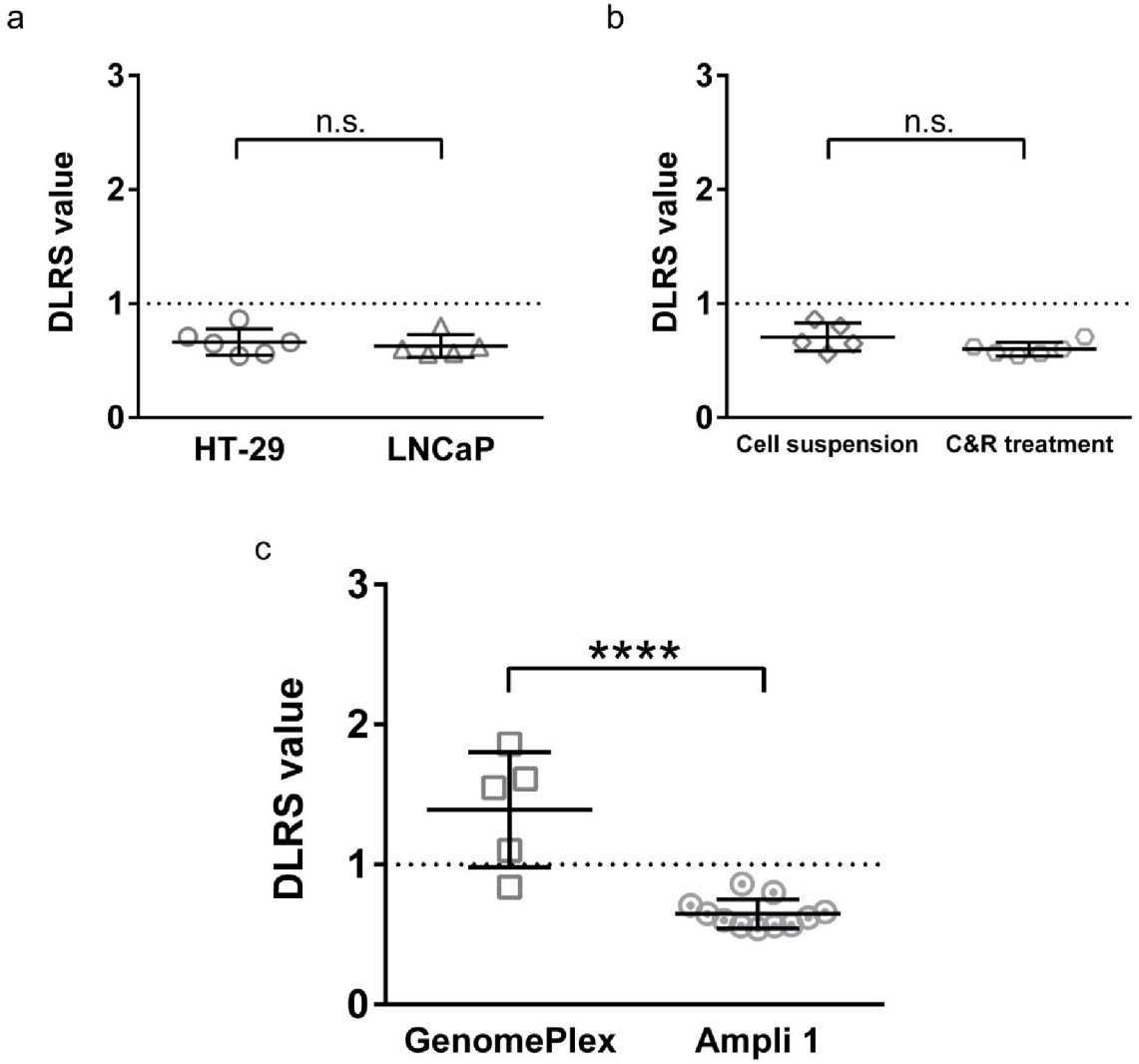

Figure 15. Quality assessment and comparison of single cell array-CGH of DNA from different cultured cancer cells amplified by *GenomePlex* or *Ampli1* [Figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]**.** The derivative log ratio spread (DLRS) to indicate noisiness in log ratio data are shown. All single-cell samples amplified by *Ampli1* show DLRS values < 1 (dashed grey line). (**a**) Single cells from both cell lines were amplified using *Ampli1*. No cell line-specific difference (unpaired t-test, two tailed, P = 0.6231) was seen at the single-cell level. (**b**) Using *Ampli1*, no difference (*P* = 0.0923) was seen between single cells directly manipulated from cell suspension and cells recovered from C&R detector. All single cells amplified by *Ampli1* have DLRS values < 1 (dashed grey line). (**c**) Single-cell samples amplified with *GenomePlex* yielded higher DLRS values compared to cells amplified with *Ampli1* (unpaired *t*-test, *P* < 0.0001). The dash line indicates the threshold value suggested for single cell. All error bars represent SD.

[The following part of result originally published by *Chen S et al.* in *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]

As a further quality control, the DNA quality of recovered single cells can be evaluated using array-CGH. WGA products of single cells amplified by *GenomePlex* and *Ampli1* were selected for array-CGH analysis based on QC-PCR. Generally, compared to unamplified gDNA (gDNA) extracted from bulk HT-29 cells (**Fig.16-i**), using both WGA methods, the profile of all representative single HT-29 cell samples shared similar gains and losses (**Fig.16**, **ii-iv**). In detail, the profile showed that with the same DNA quality (3-4 bands), less noisy signals can be seen on both the recovered and the non-C&R treated single cell amplified by *Ampli1* (Supplement **Fig.16-iii** and **iv**) than the sample amplified by *GenomePlex* (**Fig.16-ii**). Meanwhile, due to the decreased background noise, two losses on chromosome 14 and 18, and one gain on chromosome 15 detected on gDNA (**Fig.16-i**) were validated on both *Ampli1* amplified sample as well (**Fig.16-iii** and **iv**), whereas it was not indicated on *GenomePlex* amplified samples. To go into detail on HT-29 cells after *GenomePlex* (n = 4) and *Ampli1* (n = 6) amplification. *Ampli1*-processed single cells presented a higher number of aberrations in *Ampli1*-processed cells (mean 37.0 vs 12.0 Mb) and significantly shorter aberrations when compared to *GenomePlex*-processed cells (mean 14.01 vs 49.9 Mb, $P < 0.0001$; **Fig.17**).

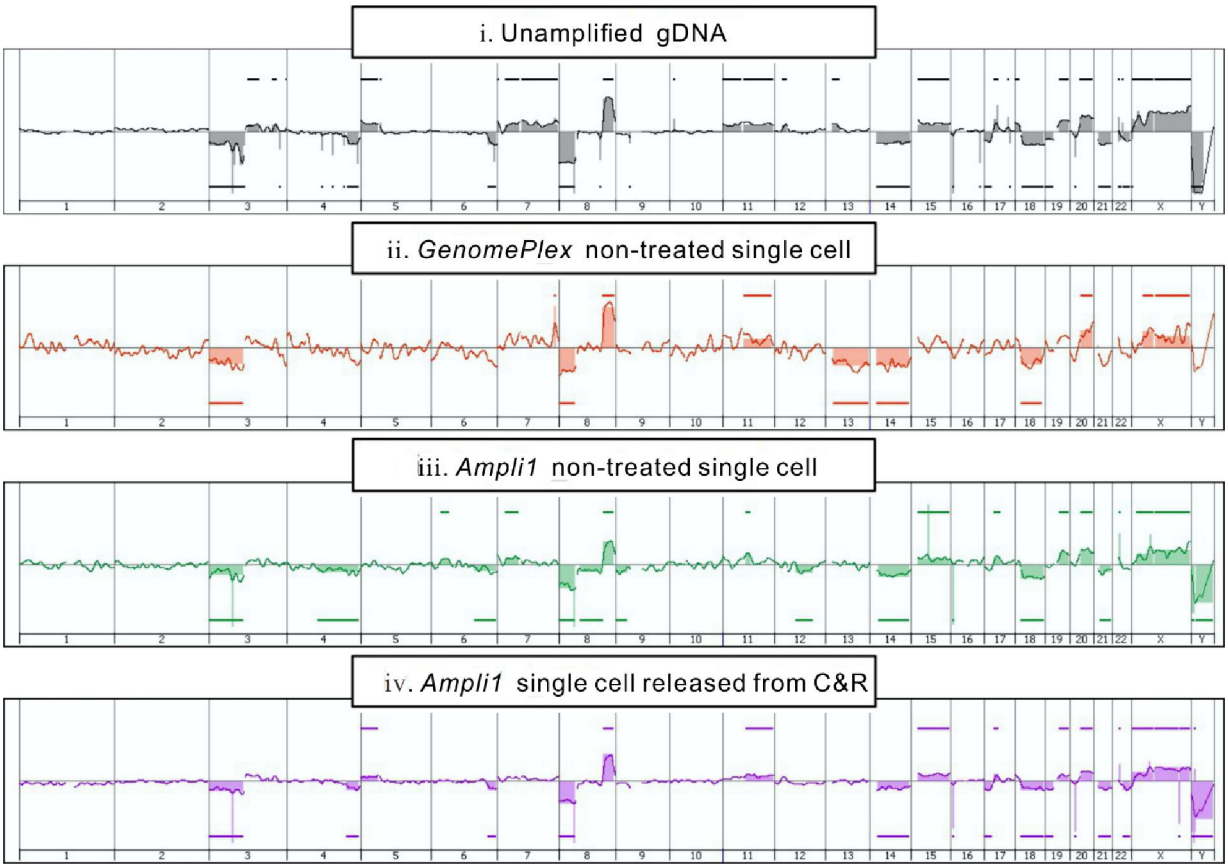

Figure 16. Genome-wide array-CGH profiles of unamplified gDNA and amplified DNA from HT-29 cells [Figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]**.** Gains and losses of unamplified gDNA (i), single cell from cell suspension amplified by *GenomePlex* (ii), single cell from cell suspension amplified by *Ampli1* (iii) and single cell recovered from C&R detector amplified by *Ampli1* (iv). The *y* axis of each profile represents log2 ratios for array CGH probes along the genome, comparing each sample to normal reference DNA. The horizontal line in the middle of each profile indicates a balance. In each profile, colored segments highlight gain (above the horizontal line) and loss (under the horizontal line) calls.

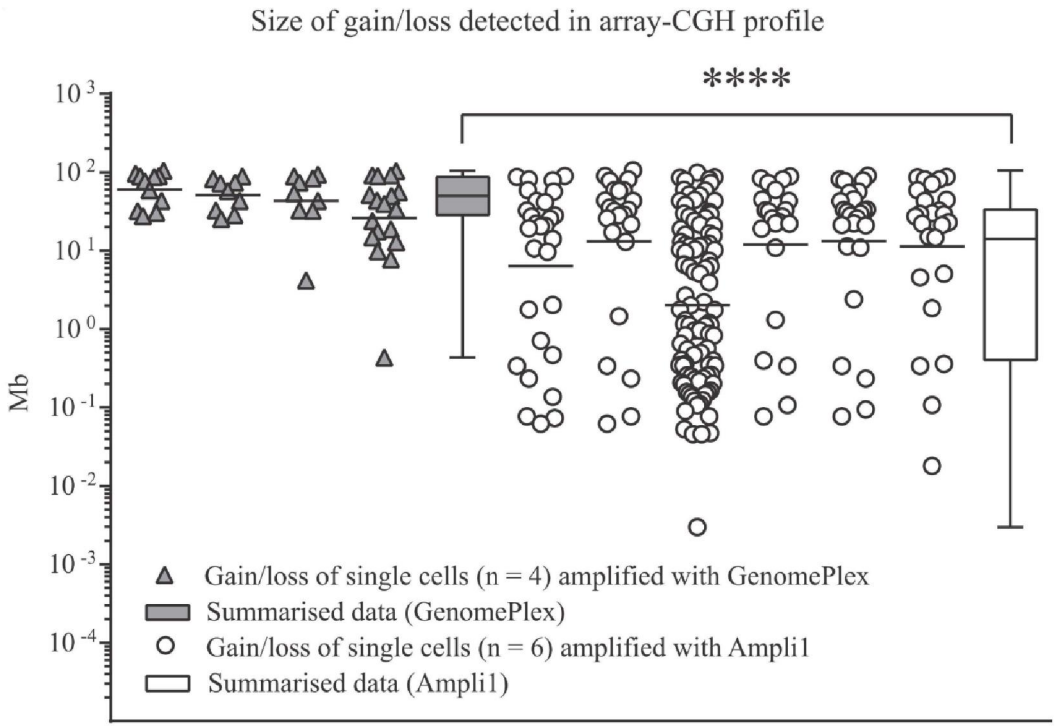

Figure 17. Quality assessment and comparison of both amplification methods based on array-CGH profiles of single-cell samples [Figure and legend originally generated by Thomas Kroneis and published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c)]**.** Single cells amplified by means of *Ampli1* (white circles) yielded a higher number of gains and losses at low sizes as compared to *GenomePlex*-amplified cells (grey triangles). This is highly significant when we 397 analyzed the aberration detection performance based on the WGA-method ($P < 0.0001$, Mann-Whitney U test) (white bar: *Ampli1*; grey bar: *GenomePlex*) used.

4.3. Array-CGH Profiles of Recovered Single Prostate Cancer Cells

To further evaluate the C&R detector, prostate cancer cell lines were used for array-CGH analysis. Untreated and released single cells as well as cell pools of LNCaP and PC-3 cell lines were micromanipulated, amplified using *GenomePlex* and *Ampli1*, and forwarded to array-CGH analysis. Circos plot was generated to reveal the chromosome aberrations of selected samples. We compared the array-CGH performance of two *Ampli1* amplified single LNCaP cells released from C&R detector to unamplified gDNA from bulk cells. The profile clearly demonstrated that WGA products of both released single cells enabled successful array-CGH. One major copy number gain

on chromosome 1q, and several major losses on chromosome 1p, 2, 6, 13 and 19 showed on the profile of gDNA (**Fig. 18**, track 1), can all be detected on released single cells (**Fig. 18**, track 3 and 4), as well as on one selected untreated single LNCaP cell (**Fig. 18**, track 2). Similar results were obtained from another prostate cancer cell line, PC-3 (**Fig. 19**) confirming the suitability of released cells for array-CGH analysis. Interestingly, we observed prevalent microdeletions in the CGH profile of single cells which were not observed in the gDNA from bulk cells (**Fig. 18 and 19**), which was partly due to the single-cell heterogeneity. Taken together, array-CGH analysis showed that DNA quality of single cell samples released from the C&R detector was not affected by the C&R procedure and suitable for molecular analysis. After optimization of WGA, several samples of single cells and cell pools of each group were forwarded to array-CGH analysis and NGS.

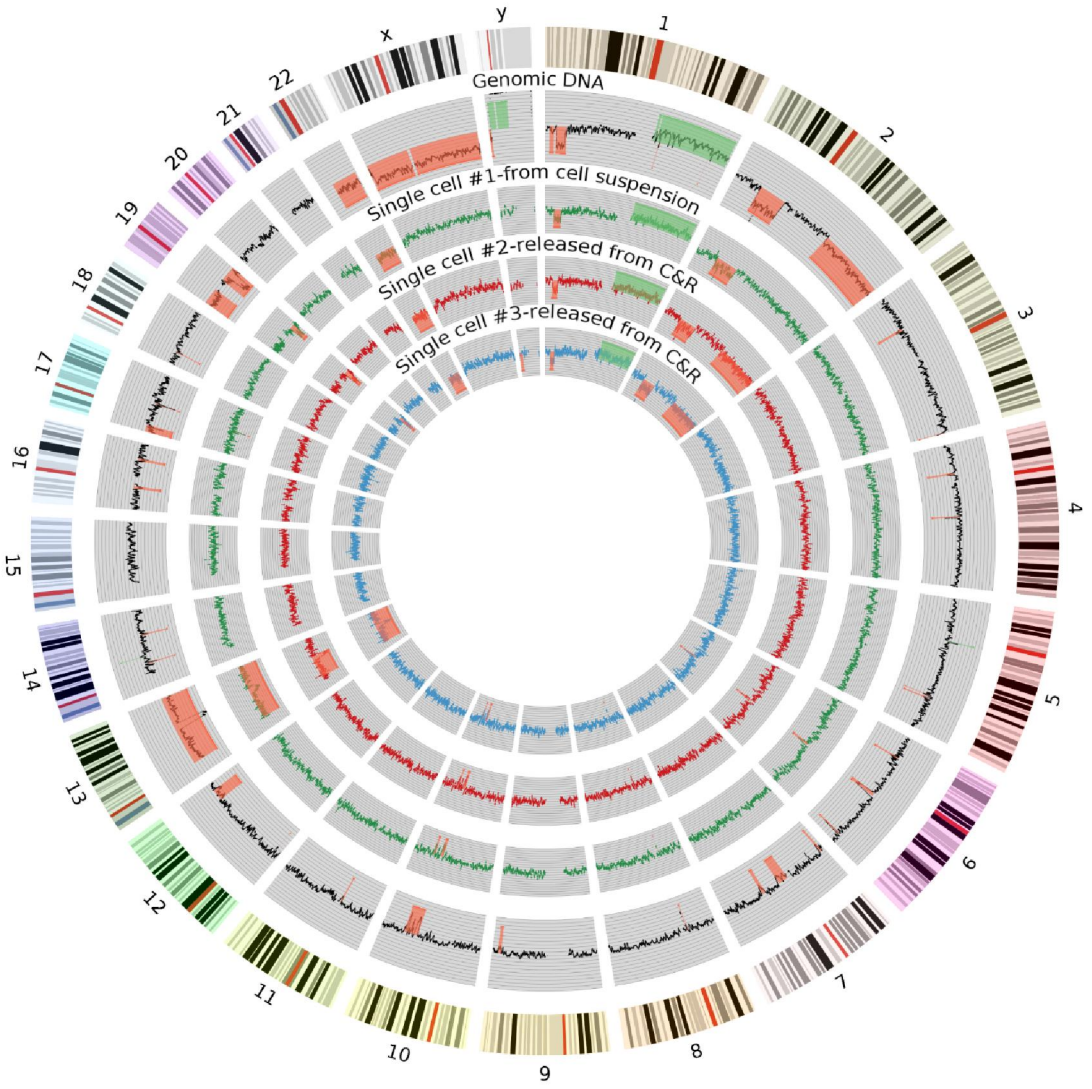

Figure 18. Genome-wide profile of copy number variations of C&R recovered single LNCaP cells (human prostate cancer cell line**) amplified by *Ampli1*** [Figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c)]**.** Circos plot was generated to depict copy number variations in the genome of LNCaP cells. The outermost ring shows the human chromosome ideogram from chromosome 1 to chromosome X oriented pter-qter in a clockwise direction (centromeres are presented in red). Other four tracks (outside-in) represent: unamplified gDNA (profile in black), single cell collected by micromanipulation directly from cell suspension (profile in green), two single cells recovered from C&R procedure (profiles in red and blue). In each profile, the middle line corresponds to no change in copy number, and data points above represent amplifications and those below represent losses. Regions of major copy number changes are highlighted in green for amplification and orange for deletion.

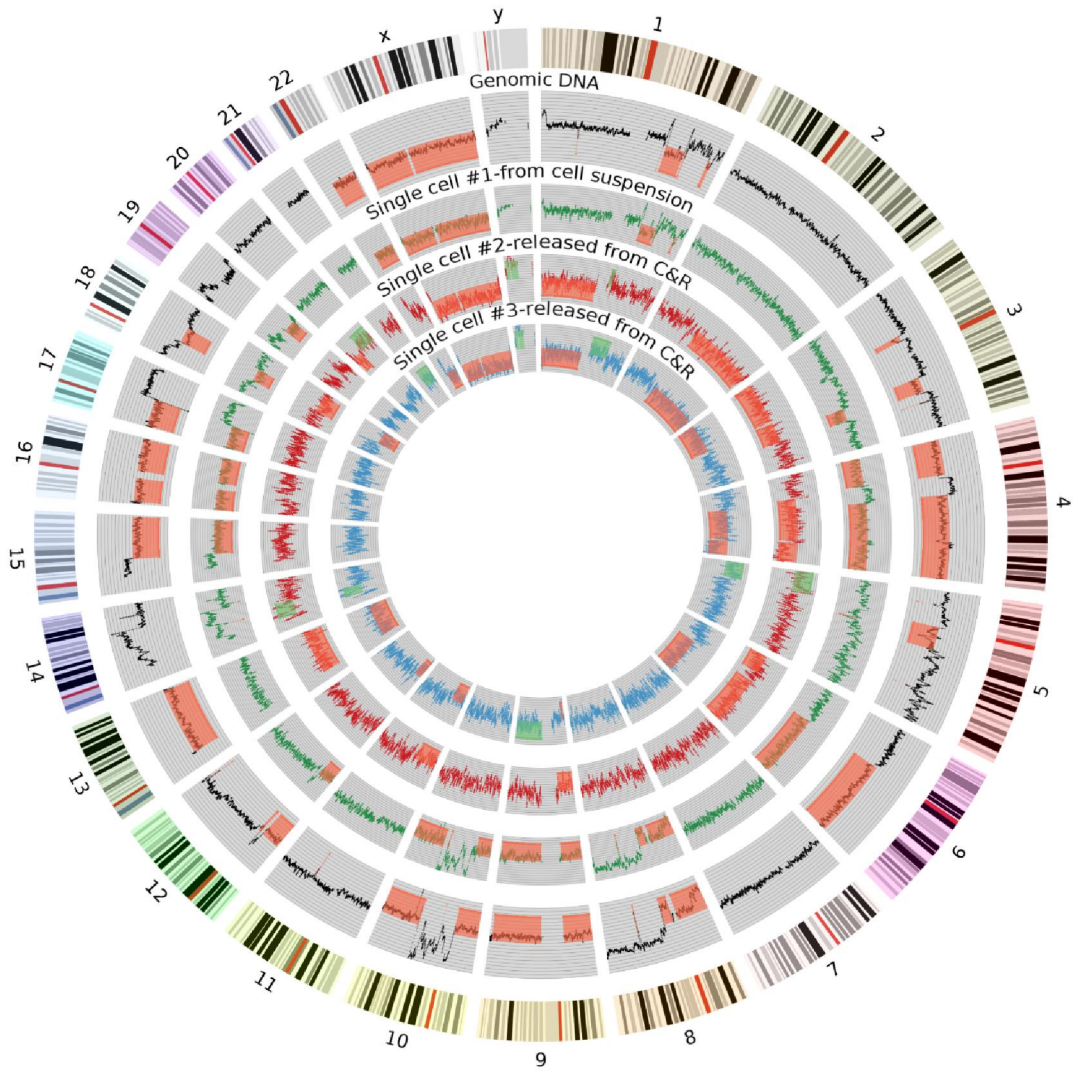

Figure 19. Genome-wide profile of copy number variations of C&R recovered single PC-3 cells (human prostate cancer cell line**) amplified by *Ampli1*** [Figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c)]**.** Circos plot was generated to depict copy number variations in the genome of PC-3 cells. The outermost ring shows the human chromosome ideogram from chromosome 1 to chromosome X oriented pter-qter in a clockwise direction (centromeres are presented in red). Other four tracks (outside-in) represent: unamplified gDNA (profile in black), single cell collected by micromanipulation directly from cell suspension (profile in green), two single cells recovered from C&R procedure (profile in red and blue). In each profile, the middle line corresponds to no change in copy number, and data points above represent amplifications and those below represent losses. Regions of major copy number changes are highlighted in green for amplification and orange for deletion.

4.4. Detection of Non-Synonymous Mutations of C&R Recovered Single Cells Using Ion Torrent-PGM Platform

To further evaluate the C&R detector for single cell analysis, WGA products of 15 recovered single cells (LNCaP and HT-29 cell line cells for 10 and 5 samples, respectively) amplified by *Ampli1* and unamplified gDNA of both cell lines with known genetic alterations were selected for targeted next-generation sequencing (NGS) analysis. Our data showed that recovered single cells performed as well as bulk DNA in NGS analysis, so we were capable of detecting targeted mutations on the single-cell level. The *Ampli* CHPCustom Beta panel (Silicon Biosystem) was designed to sequence 2265 COSMIC hot spot regions across 315 amplicons in 50 cancer-related genes (**Table 11**). Considering the bias introduced by WGA for single cells and the observed error rate of Ion Torrent PGM (1.78%) previously reported (Quail et al., 2012), the variant frequency less than 10% was filtered out. All single cell samples of LNCaP and HT-29 were sequenced with a minimum total sequence of 2,247,600 and 1,618,799, respectively. All samples were sequenced to a depth of > 1 million reads with an expected range between 90 and 130 bp and 87-93% of reads mapped to reference genome (**Fig. 20**). To validate the quality of the sequenced single cell samples, we examined the Quality Following Alignment, AQ20 score, which corresponds to the longest length at which the error rate is 1% or less and more than 90% reads were above AQ20, indicating efficient sequencing and equally good performance of both single cells and unamplified bulk DNA.

Table 11. ***Ampli1*** **CHPCustom Beta panel target gene list*** [table originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c) with minor modification]

Genes	Chromosomes	Amplicon #	Hotspot #	Genes	Chromosomes	Amplicon #	Hotspot #
ABL1	chr9	5	19	IDH2	chr15	1	12
AKT1	chr14	2	6	JAK3	chr19	3	6
ALK	chr2	1	1	KDR	chr4	6	8
APC	chr5	2	17	KIT	chr4	6	114
ATM	chr11	9	13	KRAS	chr12	3	63
BRAF	chr7	2	77	MET	chr7	3	6
CDH1	chr16	3	4	MLH1	chr3	1	1
CDKN2A	chr9	2	97	MPL	chr1	1	10
CSF1R	chr5	2	8	NOTCH1	chr9	3	20
CTNNB1	chr3	1	73	NRAS	chr1	3	35
EGFR	chr7	7	71	PDGFRA	chr4	4	26
ERBB2	chr17	4	19	PIK3CA	chr3	5	60
ERBB4	chr2	4	7	PTEN	chr10	2	55
EZH2	chr7	1	11	PTPN11	chr12	2	28
FBXW7	chr4	3	23	RB1	chr13	4	9
FGFR1	chr8	2	2	RET	chr10	2	4
FGFR2	chr10	3	5	SMAD4	chr18	4	13
FGFR3	chr4	5	17	SMARCB1	chr22	3	10
FLT3	chr13	4	30	SMO	chr7	4	4
GNA11	chr19	1	5	STK11	chr19	4	23
HNF1A	chr12	2	10	TP53	chr17	8	1147
IDH1	chr2	1	15	VHL	chr3	2	68

* Gene panel for sequencing was kindly provided by Silicon Biosystems

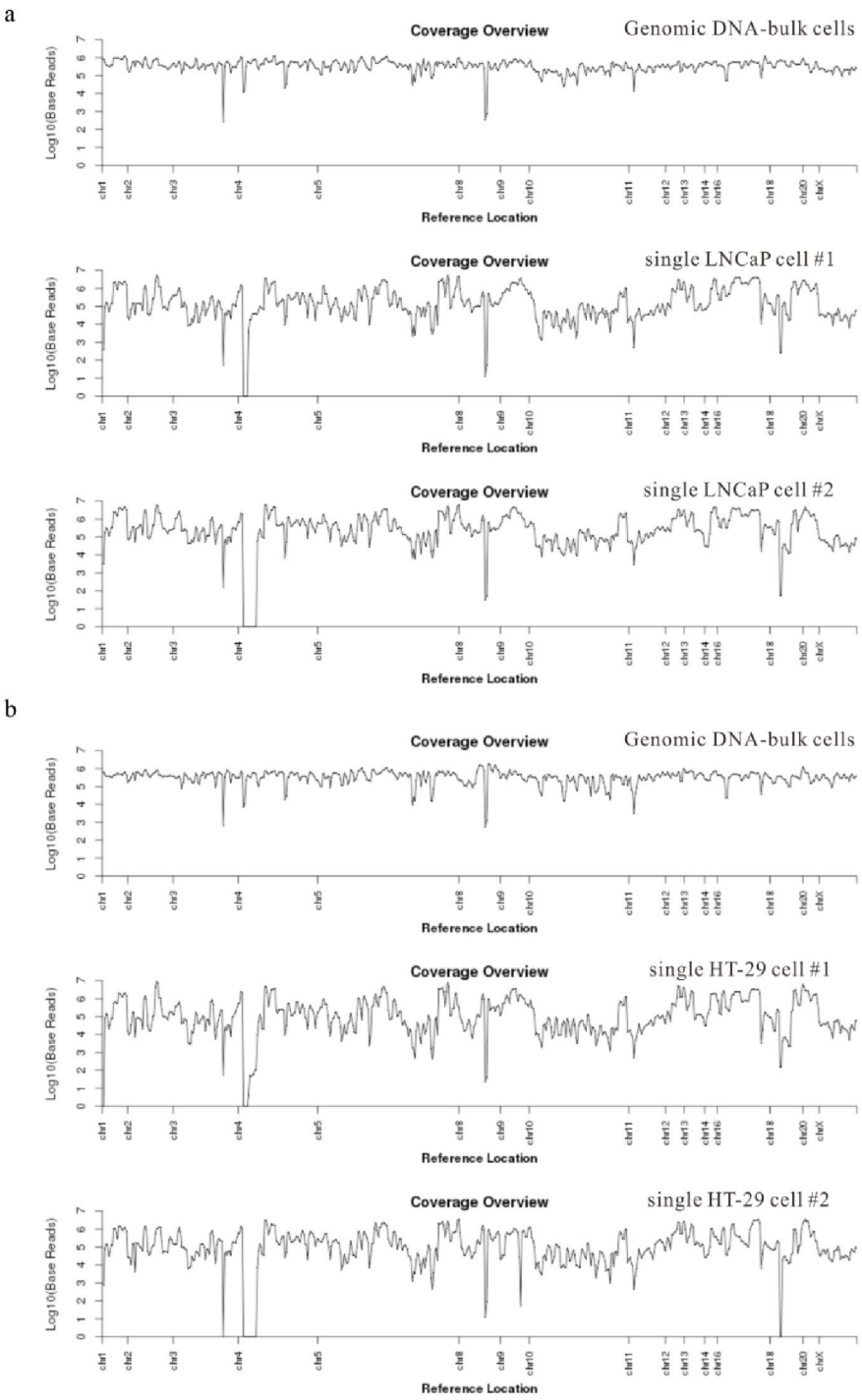

Figure 20 Quality assessments of recovered single cells from C&R procedure for NGS analysis [Figure and legend originally published by *Chen et al.* in the *Scientific Reports* (Chen et al., 2017c)]**.** (**a**) Coverage overview of gDNA (upper panel) and two recovered single LNCaP cells (middle and lower panels). (**b**) Coverage overview of gDNA (upper panel) and two recovered single HT-29 cells (middle and lower panels).

In our single cell samples, 100% of mutant allele frequency of heterozygous deletion on *PTEN* were detected on all of the ten single LNCaP cells (**Table 12**). It has been described previously that the LNCaP cells carry a frameshift mutation at codon 6, leading to PTEN mutation (Vlietstra et al., 1998), whereas our data revealed that the deletion is present on both alleles at codon 6 in our samples detected on all of the recovered single cells (**Fig. 21a**, left panel). Additionally, a heterozygous mutation of *SMAD4* was detected in 8 out of 10 recovered single cells at a mutant allele frequency range from 19.1 to 36.5% (**Table 12** and **Fig.21a**, middle panel), except two single cells, #9 and 10, which may be partly explained by the heterogeneity of single cells as well as by errors occurred during amplification. We also found single LNCaP cells that had a *TP53* mutation in one of two alleles at codon position 72 with a high frequency ranging from 91.4% to 94.3% (**Table 12** and **Fig.21a**, right panel). Detailed mutation information of each LNCaP samples is listed in **Table 12**. For HT-29 cell samples, frequently reported mutations were also detected consistently in C&R recovered single cells and in bulk DNA, including an insertion of APC gene (**Fig. 21b**, from left to right: 1st panel), a heterozygous SNP of *BRAF* gene (**Fig. 21b**, 2nd panel), and homozygous SNP of *TP53* (**Fig. 21b**, 3rd panel) and SMAD4 genes (**Fig. 21b**, 4th panel) with 100% of allele frequency across all samples (**Table 13**). Detailed mutation information of each HT-29 cell is listed in **Table 13**. Besides those amino-acid changing mutations which have been frequently reported previously, several SNPs were found only in our individual single cells with mutation frequency more than 10%. For example, in **Table 12**, single LNCaP cell #4 showed two SNPs on the gene *FBXW7* and *SMO*, which was not detected on other single cells and gDNA. Additionally, other noncoding SNPs were also detected on recovered single-cells of both cell lines (**Table 14 and 15**). Taken together, our data show that single cells isolated by C&R may be used as starting materials for detecting cancer related genetic alterations that derive from either primary or metastasis sites, and with hardly any impairment of DNA quality.

Table 12. Non-synonymus mutation frequencies of *Ampli1*-amplified single LNCaP cells after recovery from the C&R detector as well as non-amplified gDNA of LNCaP cell line cells [table originally published by *Chen et al.* in the *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]

Gene	Mutation	COSMIC ID	Mutation frequency (%)											
			Single LNCaP cells											gDNA[a]
			#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	**mean**	
PTEN	del[b]	-	100	100	100	100	100	100	100	100	100	100	**100**	100
TP53	P72R	COSM250061	92	94	93	93	92	94	91	94	93	94	**93**	95
SMAD4	L533P	COSM189744	23	30	24	20	22	37	19	22	0	0	**20**	22
CTNNB1	M12V	-	0	0	0	0	0	0	0	0	13	0	**1**	0
FBXW7	A503T	-	0	0	0	13	0	0	0	0	0	0	**1**	0
SMO	A401V	-	0	0	0	15	0	0	0	0	0	0	**2**	0

[a] gDNA, non-amplified

[b] deletion

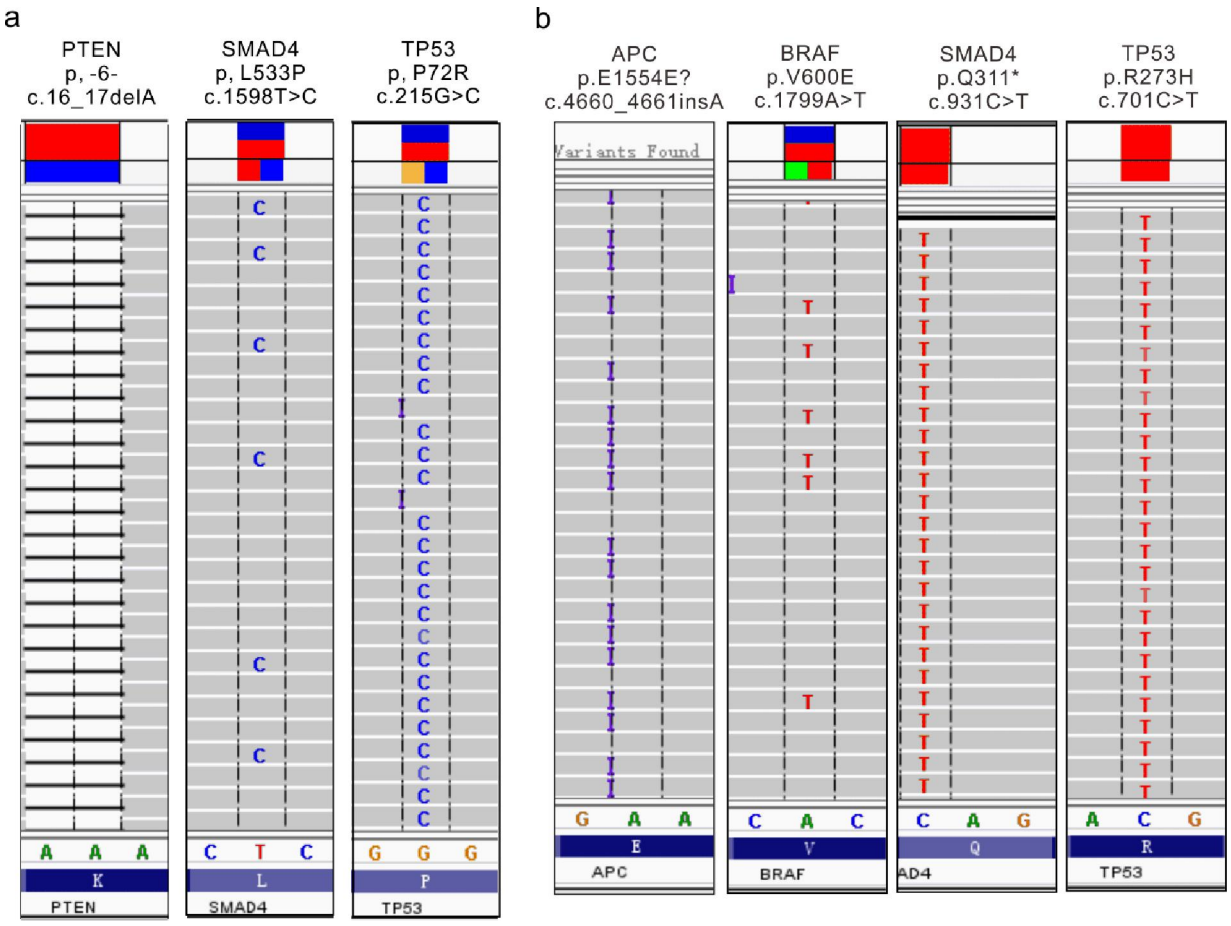

Figure 21. Selected hotspot mutation profiles of single cells recovered from C&R detector. (**a**) Integrative Genomics Viewer (IGV) visualizations show mutations in three genes of recovered single LNCaP cells. From left to right: a frameshift mutation (caused by deletion) of *PTEN* in position 87864485-87864486 of chromosome 10, a T>C mutation of *SMAD4* in position 48586262 of chromosome 18, and a G>C mutation of *TP53* in position 7577120 of chromosome 17. (**b**) IGV visualizations show mutations in four genes of recovered single HT-29 cells. From left to right: a frameshift mutation (caused by insertion) of *APC* in position 112840260-112840261 of chromosome 5, a A>T mutation of *BRAF* in position 140753336 of chromosome 7, a C>T mutation of *SMAD4* in position 51059892 of chromosome 18, and a C>T mutation of *TP53* in position 7577120 of chromosome 17. All data were aligned against hg19.

Table 13. Non-synonymus mutation frequencies of *Ampli1*-amplified single HT-29 cells after recovery from the C&R detector as well as non-amplified gDNA of the HT-29 cell line [table originally published by *Chen et al.* in the *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]

Gene	Mutation	COSMIC ID	Mutation frequency (%)						
			Single HT-29 cells						gDNA
			#1	#2	#3	#4	#5	**mean**	
KIT	M541L	COSM28026	24	27	23	24	30	**26**	26
APC	E1554E [a]	COSM33818	84	53	57	49	61	**61**	65
BRAF	V600E	COSM476	23	34	25	0	25	**21**	28
TP53	R273H	COSM10660	100	100	100	100	100	**100**	100
SMAD4	Q311 [a]	COSM14163	100	100	100	100	100	**100**	100
SMAD4	H530R	-	18	0	0	0	0	**2**	0

[a] mutated to stop codon

Table 14. Noncoding SNP frequencies of *Ampli1*-amplified single HT-29 cells after recovery from the C&R detector as well as non-amplified gDNA of the HT-29 cell line [table originally published by *Chen et al.* in the *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]

Gene	Mutation frequency						
	Single HT-29 cells (WGA: *Ampli1*)						gDNA
	#1	#2	#3	#4	#5	**mean**	
DDR2	100	100	100	100	100	**100**	100
ALK	100	100	100	100	100	**100**	100
PIK3CA	9	15	14	27	6	**14**	13
PIK3CA	60	27	40	30	29	**37**	33
FGFR3 T653	100	-*	100	-	100	**100**	100
PDGFRA P567	100	100	100	100	100	**100**	100
KIT K546	76	73	78	77	71	**75**	75
KIT L862	14	27	23	29	14	**21**	25
EGFR	70	64	43	37	59	**55**	49
EGFR	98	100	99	99	99	**99**	98
EGFR	100	100	100	100	100	**100**	100
EGFR	100	100	100	100	100	**100**	100
EGFR	100	100	100	100	100	**100**	100
EGFR Q787	57	100	46	26	66	**59**	50
SMO	99	99	99	99	99	**99**	100
RNF5P1	100	100	100	100	100	**100**	100
RET L769	68	50	71	87	80	**71**	67
INPP5F	100	100	100	100	100	**100**	100
FLT3	60	100	52	71	49	**66**	76
AR	0	28	20	61	21	**26**	32

* No data reported.

Table 15. Noncoding SNP frequencies of *Ampli1*-amplified single LNCaP cells after recovery from the C&R detector as well as non-amplified gDNA of the LNCaP cell line [table originally published by *Chen et al.* in the *Scientific Reports, 2017* (Chen et al., 2017c) with minor modification]

Gene	Mutation frequency											
	Single LNCaP cells (WGA: *Ampli1*)											gDNA
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	**mean**	
DDR2	100	100	100	100	100	100	100	100	100	100	**100**	100
ALK	47	39	72	42	58	37	60	26	65	57	**50**	47
ALK	100	100	100	100	100	100	100	100	100	100	**100**	100
PIK3CA	15	21	14	13	20	20	18	19	18	15	**17**	18
PIK3CA	56	58	60	51	62	62	50	66	60	47	**57**	50
FGFR3 T653	-*	100	-	-	100	-	100	-	-	-	**100**	100
PDGFRA P567	100	100	100	100	100	100	100	100	100	100	**100**	100
KIT K546	37	67	28	37	54	62	49	65	52	49	**50**	50
HMGXB3	100	100	100	100	100	100	100	100	100	100	**100**	100
HMGXB3	99	99	99	99	99	99	99	100	99	100	**99**	100
EGFR	41	47	79	62	45	61	41	47	29	65	**52**	44
EGFR	100	100	100	100	100	100	100	100	100	100	**100**	100
EGFR	100	100	100	100	100	100	100	100	100	100	**100**	100
EGFR	51	45	68	68	51	56	47	60	100	70	**62**	51
EGFR Q787	49	35	55	47	46	48	37	62	48	49	**48**	50
SMO	99	100	99	98	99	99	100	100	99	99	**99**	100
RNF5P1	56	67	67	57	48	42	34	83	50	53	**56**	49
FLT3	56	60	68	100	67	54	55	58	90	51	**66**	66
FLT3	58	66	50	81	62	49	51	72	57	46	**59**	66
TP53 P152	57	47	39	62	49	48	50	28	42	31	**45**	47
TP53	100	100	100	100	100	100	100	100	100	100	**100**	100
DERL3	65	48	60	59	60	72	77	0	52	60	**55**	66
AR	100	100	100	100	100	100	100	100	100	100	**100**	100

*No data reported

5. DISCUSSIONS

In this report we show our data regarding the capture efficiency of a device designed for *in vivo* harvesting of CTCs in a cohort of nonmetastatic high-risk PCa patients. Although there was no overt metastasis detected by the conventional imaging methods in all 51 patients, the presence of CTCs in the circulation were detected in some of them. This observation is consistent with previous studies showing that nonmetastatic tumors may give rise to CTCs (Davis et al., 2008, Bidard et al., 2009, Rink et al., 2011, Lucci et al., 2012) and confirms that the metastatic dissemination is an early event in the tumor progression prior to the presence of clinically apparent metastasis (Hüsemann et al., 2008, Eyles et al., 2010).

In localized cancer, CTCs are present at much lower density in the bloodstream compared to metastatic cancer (Nakagawa et al., 2007, Krishnamurthy et al., 2010). Hence, it is a great challenge for such CTC study and requires highly sensitive, reliable, and specific techniques. To determine the role of CTCs in localized cancer disease, the recovery of CTCs must be increased in order to extract meaningful clinical information. For this purpose, one could improve the sensitivity of the CTC detection assay, widen the definitions of CTC, or increase the blood volume for sampling. Currently, most of the techniques detect CTCs only *in vitro* using limited volume of blood drawn from the patient randomly at a certain time. However, whether CTCs are evenly distributed in the blood stream and continuously disseminated into blood is still under debate. To characterize CTCs from a one-time blood sample may not reflect the real status of cancer disease. In the current study we employed the DC01 for CTC detection, providing a unique advantage for identification of cells taken from directly the blood circulation continuously within 30 min, which to some extent decreases the potential bias of CTC distribution timely and spatially. Although there are only few reports regarding the use of DC01 for the study of CTCs, this method is very promising as it allows to detect CTCs directly in the circulation of patients in a simple way. By introducing the wire into a cubital vein for a 30-minutes incubation, it is estimated that 1.5 to 3 liters of blood flow past the DC01 and CTCs can be captured when they pass by, making its application superior to the others (Saucedo-Zeni et al., 2012). This method substantially increases the chance for CTC isolation compared to the gold standard technology, CellSearch which only works *ex vivo* using 7.5 mL of drawn blood.

As the CellSearch and DC01 both identify CTCs based on EpCAM positive cells, we performed a direct comparison of these two methods in the study. Our data show higher pretherapy CTC detection rates in up to 39.2% of patients compared to published data with the gold standard CTC isolation method CellSearch with 19.6% positive samples. Meanwhile, the incidence of CTC positivity of 19.6% at baseline using CellSearch was within the range of published data by other studies in nonmetastatic prostate cancer (Davis et al., 2008, Thalgott et al., 2013, Loh et al., 2014, Thalgott et al., 2015, Kuske et al., 2016). We further combined the results of CTC counts from both sampling time points which revealed that the DC01 provides a significantly increased detection rate of 33.7% compared with CellSearch of 18.6% (P = 0.024). Besides an increased CTC-positivity rate, paired analysis displayed a significant higher number of CTC counts using DC01 (P = 0.0062), showing that DC01 is a more sensitive and effective tool for CTC isolation in high-risk PCa. A similar comparison was performed by Gorges *et al.* in 2015 in lung cancer patients, and they demonstrated higher CTC detection efficiency using DC01 as well (CellSearch 27% vs. DC01 58%) (Gorges et al., 2015).

The perspectives regarding the clinical utility of CTC detection can be summarized as follows: 1) early detection of cancer progression in patients with localized cancer disease; 2) assistance with monitoring of treatment effectiveness in cancer patients; 3) detection of MRD; 4) as a surrogate endpoint for clinical trials. The clinical value of CTCs as a prognostic marker has been often reported in metastatic PCa (Moreno et al., 2005, Danila et al., 2007, De Bono et al., 2008). And many agents and targeted drugs have been developed for metastatic cancer. However, the most effective way to reduce metastasis-related cancer mortality is early detection prior to progression when the cancer still localized within its primary site. There is no doubt that early detection of cancer relapse/progression is associated with a greater range of treatment options and better prognosis (Cristofanilli et al., 2004, Hayes et al., 2006). We observed no statistical correlations between the presence of CTCs at baseline and a series of clinicopathological features which are typically used to predict cancer disease outcome, nor did we find any clinical features indicating a worse status of cancer progression in CTC positive patients. Furthermore, we found no statistical difference with respect of clinical characteristics between CTC-positive and CTC-negative patient groups. Those results are in line with and add to the current limited literature that demonstrate low levels of CTCs detected using the CellSearch system in nonmetastatic high-

risk PCa (Davis et al., 2008, Thalgott et al., 2013, Loh et al., 2014, Thalgott et al., 2015, Kuske et al., 2016), suggesting that CTCs provide prognostic information independent of currently used parameters for evaluating the cancer status.

Notably, the cancer status in several patients of our patient cohort indicated a very high risk based on conventional pathological features. They presented either with a T-stage of 3, and/or a PSA value more than 60 ng/mL, and/or a Gleason score of $\geqslant$ 9. However, all of them were found negative for CTC using CellSearch and DC01. As a potential independent prognostic marker shown by our data and previous report, CTC negativity may indicate a better clinical outcome regardless of the level of other clinical parameters. Thus, further follow-up of clinical outcomes of those patients is crucial to explore whether they can be more successfully cured with radiotherapy than those CTC-positive patients but with less severe cancer status at baseline (lower T-stage, lower PSA value, and/or less Gleason score).

Compared to single CTCs, CTC clusters may be more aggressive in forming distal metastases (Aceto et al., 2014). Several specialized platforms have been developed to target CTC clusters (Au et al., 2017, Sarioglu et al., 2015, Au et al., 2016). Although DC01 was not specially designed to capture CTC clusters, several clusters were incidentally observed in two patients in this study who presented with higher CTC numbers. Each of those cluster comprised only few CTCs (two to three CTCs in our data). Similarly, Gorges *et al.* identified CTC clusters (range from 3 to 7 cells) in 20 of 185 wires in lung cancer patients (Gorges et al., 2015). The low incidence of CTC clusters in our study might be ascribed to the early stage of cancer disease. We are unable to clarify the clinical relevance of CTC clusters in the present study.

A strength of our study was to compare the DC01 to CellSearch system with respect to the CTC-positivity rate in a cohort of 51 patients who underwent radiotherapy. Most of the patients (n = 44) had paired blood samples which may be of great value for checking whether CTC counts are a predictive marker for cancer relapse when combined with follow-up data. In the present patient cohort, the difference was not significant, however, which may due to the small sample size and the consequentially limited power of the statistical analysis. Low detection rate of CTCs may also be due to the mechanism chosen for isolation. Currently, most of the platforms make use of EpCAM to target CTCs. During cancer progression, EMT process has been well demonstrated by *in vitro* and *in vivo* studies (Kang and Massagué,

2004, Kudo-Saito et al., 2009). It has been reported that as CTCs are precursors of cancer metastasis, disseminated cancer cells (epithelial-originated) may also undergo the EMT process during transition from primary tumor mass to metastatic site and lose their cell-to-cell adhesion and acquire mesenchymal characteristics (Kasimir-Bauer et al., 2012, Yu et al., 2013, Heerboth et al., 2015). One study showed that CTCs with mesenchymal phenotype are more closely correlated with the progression or relapse of cancer disease and resistance of chemotherapy (Yu et al., 2013). Even so, most of the available techniques now still focus only on subgroups of CTCs and epithelial markers are used most frequently. In the light of considerably different sensitivities and working principles among all kinds of methods for CTC detection and isolation, it turns out to be difficult to directly compare the efficiency of each platform. However, there is no doubt that each subgroup of CTCs should be taken into consideration in order to fully characterize the cells and to acquire the information of disease progression and treatment response prior to detectable changes in clinical symptoms.

Potential limitations of the current study need to be also considered. The most significant limitation is the variability in the timing of second sampling, which may influence the evaluation of CTC status and CTC counts. We analysed CTC counts of paired samples before and after radiotherapy analyzed in parallel by CellSearch and the CellCollector DC01. We did not find a significant decline of CTC positivity detected by CellSearch and the DC01 after treatment (**Fig. 7**). A different result was revealed by Kuske *et al*. in another cohort of nonmetastatic PCa patients underwent prostatectomy for treatment. The authors found a significant decline of the CTC positive rate after surgery when using DC01 for CTC detection (Kuske et al., 2016). One possible reason for the inconsistency of these results may be based on the longer duration between first and second sampling in our procedures. In addition, as discussed above, our single-center study was limited by the relatively small sample size of patients with early stage PCa who were investigated for MRD. The very low rate of CTC positivity as well as rare CTC counts in this population particularly limited the power of statistical analyses. For the purpose of using CTCs as potential biomarker in localized PCa, larger patient cohort and long-term follow-up information are critical with respect to PSA responses, PSA progression-free survival (PSA-PFS), PFS, and OS. Meanwhile, comparative studies of potentially valuable platforms for capturing CTCs are also necessary. It remains to be further evaluated in larger patient cohort whether counting

CTCs is a useful screening tool for the early detection of cancer relapse in high-risk PCa patients.

Moving beyond the enumeration of CTCs, downstream molecular analysis of CTC is expected to help stratifying patients more precisely for targeted treatment. In prostate cancer, several genetic disorders have been frequently reported as driving events of cancer progression, drug resistance, and relapse, including AR variants (Guo et al., 2009, Sun et al., 2010), PTEN loss (Li et al., 1997, Wang et al., 2003), ETS gene rearrangement (Tomlins et al., 2005, Petrovics et al., 2005), etc. Those studies were done mainly on tissue samples from tumor entities. There is no doubt that using conventional tumor biopsies to detect cancer disease remains the gold standard method. However when it comes to metastatic tissue, it is often difficult to obtain sufficient samples for genetic analyses. In this case, liquid biopsy offers an alternative resource that can be rapidly and non-invasively acquired with much less pain, risk, and expense. Recent studies on prostate cancer demonstrated that molecular analysis of CTCs could serve as more accurate approach to predict clinical responses to hormonal therapies. One of such target is to examine androgen receptor splice variant-7 (AR-V7) mRNA of CTCs in mCRPC. It has been proven that detectable AR-V7 mRNA or protein in CTCs was associated with resistance to anti-androgen therapy with enzalutamide and abiraterone (Antonarakis et al., 2014, Steinestel et al., 2015, Scher et al., 2016). Further analysis by Antonarakis *et al.* in a cohort of 202 patients with CRPC characterized the prognostic significance of CTC-based AR-V7 mRNA detection separately in patients receiving first-line NHT and second-line NHT (Antonarakis et al., 2017). Their study also confirmed the different prognosis of patient groups categorized based on CTC–, CTC+/AR-V7–, and CTC+/AR-V7+. Recently, El-Heliebi *et al.* made use of Padlock probe technology which can visualize specific mRNA transcripts to evaluate the AR-V7 status of CTCs isolated by three devices (DC01, Parsortix and CellSearch) (El-Heliebi et al., 2018). The study allowed the quantification of CTCs without lysis of cells and easy visualization of heterogeneity of CTCs.

There is a growing recognition that intratumor heterogeneity is clinically relevant because the status of predictive biomarkers that are used for making clinical decisions can be evolved during tumor progression (Bedard et al., 2013). In recent years, the development of multiple CTC enrichment platforms together with single-cell analyses allowed for detecting gene

expression in individual CTCs and some interesting data have been revealed already. As the DC01 for clinical application at present allows only enumeration of CTCs and molecular characterization of CTCs only in bulk, compromised by interference with adhering blood cells, the potential of a novel anti-EpCAM-coated detector was evaluated by releasing cultured cancer cells bound to its surface upon *in vitro* treatment. This method facilitated subsequent molecular genetic analysis of isolated single cells allowing CTC characterization beyond mere immunohistochemical staining patterns, with potential relevance for treatment decision, which is described in the second part the present thesis. This C&R detector is also designed for *in vivo* application similar to the previous type and there are some structural improvements for enhanced capture efficiency as shown in **Table 7** (comparison of two types of wires). It is of particular note that with the help of a newly developed enzyme-containing buffer, GRB, allowing CTC isolation and single CTC analysis, thereby opening perspectives for improved clinical assessment of the cancer progression and response to treatment. In this work we show that the DNA of isolated cells is found not affected by the multiple procedures during cell detachment. First, SEM observation showed that cells remain an intact morphology when captured by the detector. Second, the DNA of recovered cells was amplifiable by WGA methods and resulted in high DNA quality allowing for further array-CGH and NGS analyses. Regarding this aspect we compared two WGA methods for downstream single CTC analysis. Our results show that the linker-adaptor based *Ampli1* WGA performed better in our settings than the fragmentation-based *GenomePlex* method. Based on the data we presented here, it is obvious that consistent matches of gains/losses and mutant hotspots were detected between C&R single cells and corresponding bulk DNA, which reveals that when this method will be applied eventually on patients for CTC isolation, the obtained single CTCs can represent the genomic profiles of its origin, either primary tumor or metastatic tumor. Using NGS as more precise analytic tool, we further noticed that single cells can be expected to acquire several unique genomic alterations due to their heterogeneity. E.g. on two single LNCaP cells, sample #9 and #10, no SNP was sequenced on SMAD4 gene compared to the mutation status of other single cells as well as gDNA (**Table 12**).

Although molecular analysis of CTCs after capture on an *in vivo* device like the DC01 can be accomplished by means of on-wire *in situ* analysis of mRNA lysed from charged detectors, for example by qPCR (our ongoing study), the unique design of the C&R provides further

options. Using cultured EpCAM-positive cancer cells, we first examined the efficiency of C&R in cell isolation/detachment, and preliminary results from several experiments showed that up to 93.16% of captured cells could be detached using the GRB. A maximum 44.25% of them could finally be recovered with good morphology and vitality in our evaluation study. As expected, detachment efficiencies of HT-29 (EpCAM high-expressing cells) and LNCaP (EpCAM low-expressing cells) cell line cells were all above 80%, suggesting that there is no difference among cells with different EpCAM expression levels. When exposed to less EpCAM-positive cells (500-50, 000) spiked into 5 mL of peripheral blood, the isolated number of cells ranged from 7 to 113 cells (**Table 9**), showing no correlation with initial spiked numbers of HT-29 cells. Towards lower target cell densities, the detachment efficiency decreased to range between > 50% and 75% (**Fig. 13A**). Meanwhile, from our *in vitro* study, we also found that the efficiency of the detector and GRB are variable due to a) type of cell line used for the experiment and to b) different cell densities applied for charging the wire. Hence, additional tests and improvements need to be done to better stabilize this platform for a reproducible and consistent efficiency. The evaluation data mentioned above were not sufficient for performing a statistical comparison as we focussed more on the downstream analysis of single released cell than on assessing the property of the detector. When making use of cells to perform WGA and array-CGH, the detached cells from the cell culture were directly transferred to the incubation procedure without fluorescent staining. There is no doubt that the less time cells are exposed to room temperature after detachment from cell culture, the better the condition of cells are suitable for downstream analysis.

We were able to perform single-cell DNA analysis after recovering LNCaP and HT-29 cells from the C&R device by enzymatically disintegrating its polymer layer. Importantly, our procedure of labelling, detaching cells from the C&R and recovering them did not affect the DNA quality of single cells cultured *in vitro* when tested after WGA and array-CGH (**Fig.14**). However, we found the DNA quality to be significantly affected by the choice of the WGA method used for single cell amplification. Different from previous data, using an optimized WGA method based on linker-adaptor amplification, almost 100% of amplified samples turned out to be of good DNA quality that are sufficient for downstream analysis. This was evident across analyzed cell lines by means of DLRS values, which reflects the noisiness in log ratio data where increased values correlate with poor signal-to-noise properties hampering accurate detection of aberrations: Single cells amplified with linker adapter-based

Ampli1 showed a >2.5-fold reduction of background noise (**Fig. 15a** and **Fig. 16**). As a consequence of this we achieved a significantly higher resolution when using *Ampli1* compared to *GenomePlex* (**Fig. 17**) probably pointing at a shortcoming of the heat-fragmentation based *GenomePlex* procedure not seen in low-template analysis of syncytial nuclear aggregates (Holland et al., 2017). Array-CGH profiles of both tumor cell lines revealed a high concordance among bulk HT-29 gDNA, micromanipulated single cells from cell suspensions, and single cells recovered from the C&R detector. Furthermore, the profiles also match with the published metaphase CGH (mCGH) profiles of the cell line (Kroneis et al., 2011) except for a few minor variations. However, each cell is unique regarding the presence of a particular variation, hence, it is rather likely that single cell profiles show differences due to heterogeneity. On the other hand, in order to process the cells biased whole-genome amplification and algorithm for analysis should be all taken into consideration for the interpretation of the data.

Using *Ampli1*-based WGA in NGS we were able to add another layer of high resolution molecular genetic analysis on top of the array-CGH screening. We detected a number of sequence variants in the single cells which matched the data obtained from non-amplified bulk cell DNA (**Table 12** and **Table 13**). Notably, the frame shift mutation in PTEN turned out to be homozygous in all our single LNCaP cells (**Table 12**), whereas it was previously reported to be heterozygous (Spans et al., 2012). Some cells showed mutations in additional genes (*CTNNB1*, *FBXW7*, *SMO*) at low frequency (range from 13 to 15%), which may be partly due to a true mutation event occurring at one allele in the hypertriploid/hypotetraploid cells or sequencing errors based on erroneous target enrichment (McCall et al., 2014).

Taken together, we provide evidence that screening for CTCs using the CE certified CellCollector DC01 allows more sensitive detection in nonmetastatic high-risk PCa compared with the CellSearch system, suggesting that in general DC01 is a promising approach for CTC detection and its *in vivo* application procedure makes it superior to other methods. Patient follow-up information is further needed in order to evaluate whether or not CTC monitoring over time (pretherapy and post-radiotherapy) allows assessment of treatment effectiveness and whether patients with higher post-radiotherapy CTC counts are associated with higher risk of cancer progression. As a proof of concept, a series of genomic analysis on released single cells, such as WGA, array-CGH, and NGS, have been carried out

using the improved C&R detector. As an effective tool, the C&R detector can be used to capture, release, and recover cancer cells effectively and makes it possible to characterize the cells on single cell level *in vitro*. Its ease of processing and the unique property of *in vivo* application makes the C&R detector an attractive technique for future clinical use.

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S.K.C, A.E-H, T.K, and P.S designed the experiments. S.K.C and A.E-H performed the experiments. G.T, T.L, M.P helped with patient enrollment, blood draws, CC application and sample fixation, clinical follow up of patients. Z.T.C, B.T helped with linker-adapter based single cell WGA, contributed reference samples for CGH. G.L helped with design and coordination of SEM experiment. K.K helped with NGS, including data processing, interpretation and discussion. S.K.C, A.E-H, T.K, P.S interpreted the data and wrote the manuscript. S.K.C, A.E-H performed experiments and analyzed the data. S.R, A.K carried out the CTC enumeration on CellSearch system. S.K.C, K.K, Z.T.C, B.T, T.K, P.S data discussion. S.K.C, A.E-H, K.K, T.K, P.S designed the study protocol, coordinated data discussion, wrote the manuscript. K.P coordinated the Transcan project. All authors reviewed and approved the manuscript.

9. LITERATURE

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10. CURRICULUM VITAE

PERSONAL INFORMATION

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Nationality	Chinese
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EDUCATION BACKGROUND

10/2013 – present	Ph.D. Molecular Medicine	Medical University of Graz, Graz, Austria
09/2009 – 06/2012	Master of Medicine	Soochow University, Suzhou, China
09/2004 – 06/2009	Bachelor of Medicine	Hebei Medical University, Shijiazhuang, China

WORK AND SCIENTIFIC EXPERIENCE

10/2013 – present	Ph.D. program;	Gottfried Schatz Research Center (for Cell Signaling, Metabolism and Aging) Unit: Cell Biology, Histology and Embryology, Medical University of Graz, Austria
02/2016 – 03/2016	Visiting researcher;	Institute of Biomedicine, Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg, Sweden
03/2015 – 04/2015	Visiting researcher;	Institute of Biomedicine, Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg, Sweden
01/2010 – 08/2012	Research Internship;	Chinese PLA General Hospital Cancer Center, Beijing, China
07/2007 – 06/2009	Clinical Internship;	Department of Anesthesiology, 4th Affiliated Hospital of Hebei Medical University, Shijiazhuang, China

AWARDS AND SCHOLARSHIPS

2015	Travel Grant-The 25th Annual Meeting of the German Society for Cytometry (DGfZ)
2014	EurocanPlatform-OECI fellowship for attending 2nd Translational Cancer Research Summer Course
2007	Excellent Students Awards, Hebei Medical University
2006	Excellent Students Awards, Hebei Medical University

EDITORIAL BOARD OF SCIENTIFIC JOURNAL

Since 2017	Journal of Cellular Toxicology and Cell Biology

FUNDED RESEARCH PROJECT

2015-2016	Project #394: Scanning Electron Microscopic Evaluations of Cells Captured by Detector for *In vivo* Enrichment of Circulating Tumor Cells Founded by: Franz Lanyar Stiftung, Medical University of Graz

CONFERENCES AND PRESENTATION ACTIVITIES

Jun. 2-3, 2016	Oral presentation	2nd International Symposium on Microgenomics 2016, Paris, France
Apr. 5-6, 2016	Invited speaker	Cambridge Healthtech Institute's Circulating Tumor Cells conference, Lisbon, Portugal
Mar. 19-21, 2016	Poster presentation	10th International Symposium on Minimal Residual Cancer, Hamburg, Germany
Oct. 7-9, 2015	Oral presentation	The 25th Annual Meeting of the German Society for Cytometry, Berlin, Germany
May 18-19, 2015	Oral presentation	Workshop on circulating biomarkers in cancer, Oslo University Hospital, Oslo, Norway
Oct. 8-11, 2014	Poster presentation	Advances in Circulating Tumor Cells (ACTC): from Basic Research to Clinical Practice, Crete, GREECE

CURRENT RESEARCH FOCUS

Molecular biology of cancer

Molecular diagnosis of cancer

Circulating tumor cells

Single cell molecular analaysis

Development of cancer biomarkers

11. LIST OF PUBLICATIONS (Sort by: date-most recent)

1. **S Chen**, A El-Heliebi, G Tauber, T Langsenlehner, M Pötscher, S Riethdorf, A Kuske, G Leitinger, K Pantel, T Kroneis, P Sedlmayr. *In vivo* detection of circulating tumor cells in nonmetastatic high-risk prostate cancer patients undergoing radiotherapy. In prepration

2. A El-Heliebi, C Hille, N Laxman, J Svedlund, C Haudum, E Ercan, T Kroneis, **S Chen**, M Smolle, C Rossmann, T Krzywkowski, A Ahlford, E Darai, G von Amsberg, W Alsdorf,F König, M Löhr, I de Kruijff, S Riethdorf, K Pantel, TM Gorges, T Bauernhofer, M Nilsson, P Sedlmayr. In situ detection and quantification of AR-V7, AR-FL, PSA and KRAS point mutations in circulating tumor cells. *Clinical Chemistry*, 2018 Jan 4. pii: clinchem.2017.281295. doi: 10.1373/clinchem.2017.281295. [Epub ahead of print]

3. A Markou, M Lazaridou, P Paraskevopoulos, **S Chen**, M Świerczewska, J Budna, A Kuske, TM Gorges, SA Joosse, T Kroneis, M Zabel, P Sedlmayr, C Alix-Panabières, K Pantel, ES Lianidou. Multiplex gene expression profiling of in-vivo isolated circulating tumor cells in high-risk prostate cancer patients. *Clinical Chemistry*, 2017, 51(11): 2219-2219. DOI: 10.1373/clinchem.2017.275503

4. **S Chen**, A El-Heliebi, T Kroneis. Biological and Molecular Characterization of Circulating Tumor Cells: A Creative Strategy for Precision Medicine? *Advances in Clinical Chemistry*-Volume 82. Elsevier Science Publishing, 2017 Oct. Online, doi: 10.1016/bs.acc.2017.06.00

5. A El-Heliebi, E Heitzer, T Kroneis, **S Chen**, C Haudum, J Fuchs. Potential and Challenges of Liquid Biopsies[M]//*Mechanisms of Molecular Carcinogenesis*–Volume 2. Springer International Publishing, 2017: 233-261.

6. **S Chen**, A El-Heliebi, G Tauber, T Langsenlehner, M Pötscher, K Kashofer, Z T. Czyż, B Polzer, S Riethdorf, A Kuske, G Leitinger, K Pantel, T Kroneis, P Sedlmayr. Catch and Release: rare cell analysis from a functionalised medical wire. *Sci Rep*. 2017; Online, doi:10.1038/srep43424

7. A El-Heliebi, **S Chen**, T Kroneis. Heat-Induced Fragmentation and Adapter-Assisted Whole Genome Amplification Using *GenomePlex*® Single-Cell Whole Genome Amplification Kit (*GENOMEPLEX*). *Methods Mol Biol*. 2015; 1347: 101-109.

8. A El-Heliebi, **S Chen**, T Kroneis. Using Multiplex PCR for Assessing the Quality of Whole Genome Amplified DNA. *Methods Mol Biol*. 2015; 1347: 119-128.

9. T Kroneis, **S Chen**, A El-Heliebi. Low-Volume On-Chip Single-Cell Whole Genome Amplification for Multiple Subsequent Analyses. *Methods Mol Biol*. 2015; 1347: 245-261.

10. F Bu, X Liu, J Li, **S Chen**, X Tong, C Ma, H Mao, F Pan, X Li, B Chen, L Xu, E Li,G Kou, J Han, S Guo, J Zhao, Y Guo. TGF-β1 induces epigenetic silence of TIP30 to promote tumor metastasis in esophageal carcinoma. *Oncotarget*. 2015 Feb 10;6(4):2120-33.

12. APPENDIX

12.1. Protocols

12.1.1. Staining of the CellCollector DC01.

Operator: _____Date: _____Sample No.: _____

Sampling date	Patient ID	Sampling date	Patient ID

Reagents, Materials and Equipment

	Chemical	Storage
1	PBS (GIBCO), pH 7.2-7.4 (w/o Mg^{2+}/Ca^{2+}),	4°C storage
2	BSA (bovine serum albumine, Sigma)	4°C storage
3	Triton X-100	RT storage

	Solution	Component	Final concentration	Diluted/ Prepared	Storage
1	Permeabilization	Triton X-100	0.1%	PBS	1 month; 2-8°C
2	Blocking buffer	BSA	3%	PBS	1 month; 2-8°C
3	Washing buffer	PBS	1 ×	--	--

	Antibody & Hoechst	Dilution ratio	For one sample (µL)	
1	CD45-A647	1:25	16	363 µL blocking buffer
2	anti-Pan-Cytokeratin A488 (1, 2, 3, 4, 5, 6, 7, 10, 14, 15, 16, 19)	1:50	8	
3	panCK-A488 (4, 5, 6, 8, 10, 13, 18)	1:50	8	
4	anti-PSA (EPISPOT self-labeled A555)	1:80	5	
5	Hoechst 33342 (10mg/mL), diluted, 1:100 in PBS	1:50	8	392 µL 1×PBS

Other equipment	
Tweezers	0.3 mL micro glass tubes, 4×
Pipette tips / Pipet controller	Vortex-Genie 1 touch mixer
Timer	Rack
1.5 mL reaction tubes, 5×	Ice / ice box

- **Procedure**

☐ 1. *Clean:* Bring the DC01* to RT, remove the yellow screw** and use distilled water clean the non-functional part the wire to remove the visible blood residual on the surface. Label a new screw with patient code.

☐ 2. *Permeabilization*: 400 µL permeabilization buffer filled in a micro glass tube; incubate the fixed DC01 for 10 min (+2 min) at RT;

☐ 3. *Wash*: dip the DC01 in 1 mL of washing buffer **3** times (3-5 sec) in 1.5 mL tube;

☐ 4. *Blocking:* 400 µL blocking buffer filled in a micro glass tube; incubate the permeabilized DC01 for 30 min (+10 min) at RT; prepare antibody staining solution and keep in dark on ice;

☐ 5. *Wash*: dip the DC01 in 1 mL of washing buffer **3** times (3-5 sec) in 1.5 mL tube;

☐ 6. *Cell staining*: 400 µL of antibody staining solution filled in a micro glass tube; incubate the blocked DC01 for 1 h (+10 min) at RT in the dark place;

☐ 7. *Wash*: dip the DC01 in 1 mL of washing buffer **3** times (3-5 sec) in 1.5 mL tube;

☐ 8. *Nucleus staining*: 400 µL of DNA staining solution filled in a micro glass tube; incubate the stained DC01 for 5 min (+10 min) at RT;

☐ 9. *Wash*:

- Dip the DC01 in 1 ml of washing buffer **3** times (3-5 sec) and keeping the DC01 in the buffer for further 5 min;
- Put the DC01 in a new 1.5 ml tube for 1min for air dry;
- Store the wire in a new glass container until the microscopic evaluation (**1-30 min** at RT in the 1×PBS buffer; up to **2 hours** at 2-8°C in the 1×PBS buffer or store it in DRY condition at -20°C for **max. 1 week** until evaluation).

* Before IF staining, the DC01 can be stored at **-20**°C **for max. 1 month** and **-80**°C **for max. 3 months**.

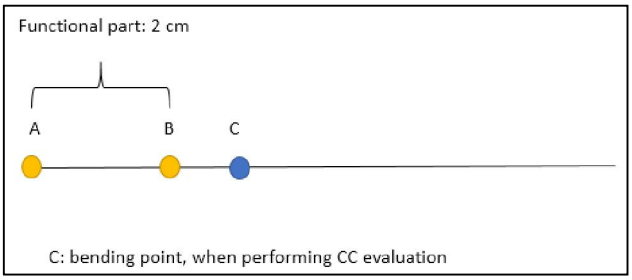
** Clean the yellow screw and glass container for further use:

- Screw: soak in water for 2-3 days, rinse to remove the blood residual, rinse with 70% ethanol, and air dry;
- Glass container: rinse with distilled water twice, rinse with 70% ethanol, and air dry.

12.1.2. Evaluation of the stained CellCollector DC01

a. Evaluation protocol:

1. Switch on the Zeiss microscope and fluorescent;
2. Program selection, e.g.: “ZEN-Shukun CTC”;
3. Remove the yellow stopper;
4. Bend the detector at the point “C” and fix it with double-side adhesive tape and normal tape on a glass slide (if necessary, clean the slide with alcohol and water before use)



5. Evaluation
   (1) Selection of 10-fold objective (20-fold also possible)
   (2) Position the DC01 centrally on the microscope table
   (3) Adjust final position of the area to be evaluated (e.g. 2 cm point) under the objective
   (4) Focus DC01 with the DAPI channel
   (5) Start the detection (see also acceptance criteria) in the whole field of view:
      a) Search with the DAPI channel for blue, nuclei-like targets
      b) If positive, then verify in the FITC channel to make sure that the expression of cytokeratin (CK) is available
      c) If the CK expression exists, then check in the Cy5 channel to see the negativity/positivity in red signal
      d) If necessary, document discovery
      e) If necessary, examine / document at a higher magnification
      f) Review of the discovery: it has to fulfill the acceptance criteria
   (6) Slowly advance the DC01 towards the tip
   (7) Review the field of view (only the cells in the center) and avoid duplicated counting
   (8) If the tip of the DC01 is reached, then turn 180° of the DC01, and repeat the above steps to check the other side of the DC01

b. Acceptance criteria

Staining outcomes:

Positive for CK Signal in the FITC channel;

Positive for PSA Signal in Cy3 channel;

Positive for DNA (Hoechst) Signal in the DAPI channel;

Positive for CD45 Signal in the Cy5 channel.

Criteria for the identification of CTCs:

The cells must possibly have an intact morphology.

The cells can be polymorphic (large cell bodies, irregular cell shapes, several cells together / piles etc.)

Nuclear staining must be positive (Hoechst positive)

The cells have to be negative for CD45.

The cells have to be positive for CK and / or PSA

The cell diameter should be $\geqslant$ 4 µm.

Nuclear staining must be clearly distinguishable from CK/PSA staining.

Unclear findings inter alia, if:

1) A conspicuous cell morphology with a large nucleus (typical for CTCs), but

Entirely without any fluorescence signal for CK/PSA, CD45 or

Multiple positive (CK/PSA and CD45 positive)

2) Objects with the size of a cell, which lie directly in or on a large artifact or blood clot

c. Storage:

For a further investigation of the stained DC01, it can be stored in a tightly closable tube in which the coated area of the DC01 will not be damaged (for example: in a 15 mL centrifuge tube; the glass tube delivered as packaging vial; optionally: cut off the coated area of the DC01 and store it in a 2 mL cryogenic vial).

The DC01 should be stored in dry condition and protected from light at -20°C (up to 3 months) or for longer storage the DC01 can be kept at -80°C.

d. Evaluation form

Date of Evaluation		Patient ID	Date of Staining	Date of Sample	CTC counts	Notes
CTC No.	Exposure time (ms)					
	CK -/+	PSA -/+	Nucleus -/+	CD45 -/+	File name	Notes
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

12.1.3. Immunofluorescent staining of cells

Date: _____ Operator: _____ Cell line: _____ Cell density: _____

(Always keep the C&R in WET condition and AVOID DRYING)

[Reagent preparation (before start cell culture)]

- 2% BSA buffer (for dissolution, vortex shortly first and mix on vertical roller for 10-20 min):
    a. 1*PBS (cell culture use) 40mL
    b. BSA 0.8g
- Rinse a EDTA tube with 2 mL of 2% BSA buffer shortly and discard the rinse buffer;
- Block tube wall surface with 5 mL of 2% BSA, 30 min, on a flat roller mixer at RT (at the speed 15 rpm)

[Cell pre-staining]

- Cell dissociation with Accutase;
- Add medium and centrifuge;
- Add PBS and centrifuge (⊙) for washing;
- Dilute 1 µL CFSE/DMSO (5 mM) in 1 mL PBS (≙ CFSE solution); pre-warmed at 37°C
- Resuspended cell pellet in 500 µL CFSE solution
- 15 min, 37°C incubation (water bath)
- ⊙ 300 g, 3 min, RT
- Resuspended in 1 mL pre-warmed medium, 30 min, 37°C regeneration (water bath)
- ⊙ 300 g, 3 min, RT
- Resuspended pellet in 1 mL Hoechst 33342 (stock 1:100 diluted in PBS; final work dilution = 1:10, 000), 10 min, 37°C (water bath)
- Add 4 mL PBS and thoroughly mix by pipetting
- Cell counting (10 ul cell suspension + 10 ul Trypan blue)
- ⊙ 300 × g, 3 min, RT
- Pellet re-suspended in whole blood or 2% BSA to adjust the cell density to ____ × _____ cells/mL (in the whole blood or BSA buffer)
- Proceed to loading steps

12.1.4. C&R treatment of pre-stained cells

Date: _____ Operator: _____ Cell line: _____ Cell density: _____

(all of the cell counting steps in this protocol are performed using Zeiss microscope in FITC channel)

[Loading cells onto C&R detector]

- Add 5 mL cell suspension (stained) along the inner wall per tube (pre-coated; AVOID air bubbles)
- Insert the C&R to the rubber cap with the help of syringe needle (20G) and immerse the 4 cm of functional part into cell suspension
- Rolling incubation at 5 rpm, RT for 30 min (support the mixer to give it a tilt)
- Rinse C&R in 5 mL of 1 × PBS until cell counting (in a 15 mL centrifuge tube)

[1st Cell counting] --- cell number after loading

- Draw a rectangle (4.5 cm × 1 cm) on a new glass slide with a hydrophobic barrier pen;
- Cut the C&R to approx. 8 cm and bend the end a bit for locating position
- Label the bending end with tape and place it immediately onto the marked glass slide to keep the functional part within the frame of the rectangle;
- Add 100-200 µL of PBS to immerse the functional part to avoid drying, adjust the position with tweezers and perform cell counting from the bottom to the tip of the functional part in FITC channel (always count the bright cells as there may be reflection signal)
- Turn 180° and repeat the counting of the other side
- Place the C&R back to the PBS buffer
- Switch off the florescence only for later use

[Cell detachment using GRB enzyme buffer]

After cell attachment (best within 4 h after cell binding)

- Preparation of GILUPI Release Buffer working solution
  - Adjust solution to a concentration of 4 mg/mL in PBS (for one sample, at least 2 mL of

buffer is needed)

- Sterilize by filtration (0.2 μm) to remove particles
- Pre-heat GILUPI Release Buffer working solution to 37°C (max. 5 min, water bath)

• Cell detachment

- Remove the cap of a 1.5 mL Epi tube and fill 1.6 mL of pre-warmed GRB
- Insert the C&R to a stopper with the help of syringe needle (20G) and immerse the functional part to 1.6 mL of GRB.
- Put tube with detector in water bath at 37°C for 20 min.
- Transfer tube with detector and shake (500 rpm) for 15 min (RT)
- ⊙ 300 g, 10 min, RT
- Remove detectors and store in PBS at 4°C (in the fridge) for a 2nd cell counting
- ⊙ 300 g, 10 min, RT
- Reduce volume until approx. 100 μL and keep the tube at 4°C (in the fridge) for a 3rd cell counting
- (when the detached cells are used for further molecular analysis): add 1 mL of PBS and resuspend the cell pellet for washing
- ⊙ 300 g, 10 min, RT
- Reduce volume until approx. 100 μL and keep the tube at 4°C (in the fridge) for single cell manipulation

[2nd Cell counting] --- remaining cells on the C&R to calculate releasing cells

• Perform the cell counting as steps in **[1st Cell counting]**

[3rd Cell counting] --- recovered cell No.

• Pipette the remained suspension and transfer the suspension onto a 16-well chamber slide for counting

• Start counting after waiting for approximately 5 min to let the cells down to the bottom.

12.2. Permission Requests from Authors

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