DEFORMABILITY-BASED SEPARATION AND SINGLE-CELL SEQUENCING OF CIRCULATING TUMOR CELLS IN PROSTATE CANCER

by

Emily Sunyoung Park

B.A.Sc., The University of British Columbia, 2008

M. Sc., Seoul National University, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Biomedical Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2017

© Emily Sunyoung Park, 2017

Abstract

Circulating tumor cells (CTCs) are malignant cells shed from a primary tumor into the bloodstream, where they have the potential to seed metastases responsible for >90% of all cancer related deaths. CTCs are particularly interesting for prostate cancer because metastases occur predominantly bone tissue, which makes biopsies difficult and low yielding. Since CTCs are accessible from peripheral blood, these cells represent a potential source of highly relevant tumor materials, which could be used to reveal new biomarkers for monitoring disease progression and evaluating drug efficacy. A key challenge in CTC isolation and characterization has been their extreme rarity in blood and their cell-to-cell heterogeneity. Both of these issues suggest the need to develop robust methods to isolate and analyze individual CTCs.

This dissertation presents a new workflow to isolate, extract, and sequence single CTCs from patients with metastatic prostate cancer. Initially, we investigated the morphology of CTCs from patients with prostate cancer, and observed that CTCs and leukocytes were similar in size, but distinct in nucleus-to-cytoplasm ratio, which suggests the potential to separate CTCs based on deformability. Based on this result, a microfluidic device that separates CTCs based on cell deformability, as well as an accompanying analytical pipeline to identify CTCs using immunofluorescence, were developed, optimized, and tested. This workflow was used to successfully enumerate CTCs from 20 patients with metastatic castrate resistant prostate cancer, as well as 25 patients with localized prostate cancer. For the former cohort, we compared our process against existing technology and demonstrated 25× greater yield. We then developed a process to isolate single CTCs using laser capture microdissection for genome sequencing. Using this process, we enriched and isolated 30 single CTCs from 3 patients with metastatic prostate

ii

cancer, and sequenced 5 of these single CTCs from a patient with matched cell-free DNA. The sequencing data confirmed the presence of major driver mutations, including PTEN and TP53, as well as heterogeneous characteristics of individual CTCs. These results demonstrate the potential of our single cell sequencing workflow to discover clinically relevant mutations from single CTCs that may aid in monitoring disease progression and guiding treatment.

Lay Summary

The majority of cancer deaths are caused by metastasis, or the spread of cancer from its original site to other tissues. For cancer to spread, malignant cells known as circulating tumor cells (CTCs), must escape from the primary tumor and circulate though the bloodstream before invading other tissues. Thus, CTCs are considered as the seeds of metastasis that could provide valuable information regarding the status and treatment options for each patient's disease. Obtaining information from CTCs, particularly genetic mutations, is challenging because CTCs are extremely rare in blood. To overcome this challenge, we developed a technology to first enrich CTCs from whole blood, and then isolate individual cells for genome sequencing. Using this process, we obtained sequence data of individual CTCs from patients with advanced prostate cancer, which revealed the presence of clinically relevant mutations that can potentially be used to evaluate disease status and help clinicians select appropriate treatment.

Preface

The research presented in this thesis is the original work of the author. The work was supported by Canadian Institute of Health Research, Natural Science and Engineering Research Council of Canada, Prostate Canada, and the Engineering-in-Scrubs training program at University of British Columbia. The research requires blood donation from healthy donors and patients with prostate cancer. The approval was obtained from the University of British Columbia ethic committee (H13-00870).

A version of Chapter 4 has been published in S. Park, R. R. Ang, S. P. Duffy, J. Bazov, K. N. Chi, P. C. Black, P. C. Black, and H. Ma. "Morphological Differences between Circulating Tumor Cells from Prostate Cancer Patients and Cultured Prostate Cancer Cells," PLoS ONE, vol. 9, issue 1 (2014). I conducted all the experiments, and wrote the manuscript together with Dr. S. Duffy and Dr. Hongshen Ma. Richard Ang developed the software to analyze the images from CellSearch[®] system.

Chapter 5 describes the microfluidic ratchet mechanism for cell separation. An original version of this model was developed by Dr. Hongshen Ma. The early modeling and proof-of-concept experiments (version 1.0) were conducted by Quan Guo and Sarah McFaul. The continuous flow ratchet device described in Section 5.2.3 was developed with Chao Jin. The testing and validation of the continuous flow device (version 2.0) were performed by me and Chao Jin. the multiplexed inlet microfluidic device (version 2.1) described in Section 5.6.2 was developed by Justin Yan. The testing and validation of the bifurcated device were performed by me and Justin Yan.

v

Section 6.3.3 describes the cell encapsulation using a hydrogel. An initial material testing was conducted by Jeong Hyun Lee. Here, I adopted this material to encapsulate cells for single-cell extraction using laser capture microdissection system. Section 6.4.4 describes the CTC enumeration software, developed by Richard Ang. Section 6.5 describes the validation of single cell analysis work using Sanger sequencing and the targeted next generation sequencing, performed by the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre. A version of this chapter is currently being prepared for publication entitled, 'Single-cell sequencing of circulating tumor cells using hydrogel encapsulation and laser capture microdissection.'

A part of Chapter 7, related to castration-resistant prostate cancer, has been published in E.S. Park, C. Jin, Q. Guo, R. R. Ang, S. P. Duffy, K. Matthews, A. Azad, H. Adbi, T. Todenhöfer, J. Bazov, K. N. Chi, P. C. Black, P. C. Black, and H. Ma. "Continuous flow deformability-based separation of circulating tumor cells using microfluidic ratchets," Small, vol. 12, pp1909-19 (2016). Another part of Chapter 7, related to localized prostate cancer, has been published in T. Todenhöfer, E.S. Park, S. P. Duffy, X. Deng, C. Jin, H. Abdi, H. Ma, and P. C. Black. "Microfluidic enrichment of circulating tumor cells in patients with clinically localized prostate cancer," Urologic Oncology, vol.34 (2016). I conducted blood sample preparation, CTC separation and identification as well as single cell extraction procedure. The targeted sequencing was performed by Kevin Beja for sample preparation and Matti Annala for sequencing data analysis in the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre. Patient characteristics and correlation of CTC counts with pathologic risk parameters for localized prostate cancer samples were prepared and analyzed by Tilman Todenhöfer. The manuscript

vi

published in Small were written by Emily Park, Simon Duffy, and Dr. Hongshen Ma. The manuscript published in Urologic Oncology were written by Tilman Todenhöfer.

Table of Contents

Abstract	ii
Lay Summary	iv
Preface	V
Table of Contents	viii
List of Tables	XV
List of Figures	xvi
List of Abbreviations	xxvi
Acknowledgements	xxviii
Dedication	xxix
Chapter 1: Introduction	1
1.1 Significance and Motivation	1
1.2 Research Objectives	3
1.3 Organization of the Thesis	3
Chapter 2: Background	5
2.1 Metastasis and Circulating Tumor Cells (CTCs)	5
2.2 CTCs in Prostate Cancer	
2.2.1 CTCs in Castration-Resistant Prostate Cancer (CRPC)	9
2.2.2 CTCs in Localized Prostate Cancer	13
Chapter 3: Literature Review	15
3.1 CTC Separation Methods	15
3.1.1 Affinity-based Enrichment	15
3.1.1.1 Positive Selection	16 viii

3.1	1.2 Negative Selection	. 22
3.1.2	Biophysical Property-based Enrichment	. 23
3.1	2.1 Size and Deformability of Tumor Cells	. 23
3.1	2.2 Microfiltration	. 25
3.1	2.3 Hydrodynamic Sorting	. 28
3.1	2.4 Dielectrophoresis (DEP)	. 32
3.1.3	Summary	. 35
3.2 S	ingle Cell Isolation Techniques	. 35
3.2.1	Fluorescence Activated Cell Sorting (FACS)	. 36
3.2.2	Micromanipulation	. 37
3.2.3	Laser Capture Microdissection	. 37
3.3 I	ONA Sequencing of Single CTCs	. 40
Chapter 4	Morphology Study of CTCs in Prostate Cancer	45
4.1 I	ntroduction	. 45
4.2 A	Approach	. 46
4.3 E	Experimental Section	. 46
4.3.1	Sample Preparation and Cell Culture	. 46
4.3.2	Isolation and Enumeration of CTCs by CellSearch Assay	. 47
4.3.2 4.3.3	Isolation and Enumeration of CTCs by CellSearch Assay Imaging Processing for Cell Size and Shape Measurement	. 47 . 47
4.3.2 4.3.3 4.3.4	Isolation and Enumeration of CTCs by CellSearch Assay Imaging Processing for Cell Size and Shape Measurement Size Measurement Calibration	. 47 . 47 . 51
4.3.24.3.34.3.44.3.5	Isolation and Enumeration of CTCs by CellSearch Assay Imaging Processing for Cell Size and Shape Measurement Size Measurement Calibration Nuclear Cytoplasmic Ratio Measurement	. 47 . 47 . 51 . 51
 4.3.2 4.3.3 4.3.4 4.3.5 4.3.6 	Isolation and Enumeration of CTCs by CellSearch Assay Imaging Processing for Cell Size and Shape Measurement Size Measurement Calibration Nuclear Cytoplasmic Ratio Measurement Sample Selection	. 47 . 47 . 51 . 51
4.3.2 4.3.3 4.3.4 4.3.5 4.3.6 4.4 F	Isolation and Enumeration of CTCs by CellSearch Assay Imaging Processing for Cell Size and Shape Measurement Size Measurement Calibration Nuclear Cytoplasmic Ratio Measurement Sample Selection	. 47 . 47 . 51 . 51 . 51

4.4.1	Cell Size	52
4.4.2	Cell Shape	55
4.4.3	N/C Ratio	57
4.4.4	Cell Shrinkage	58
4.5 S	ummary	58
Chapter 5:	Development of CTC Separation Process and Analytical Pipeline	.60
5.1 Iı	ntroduction	60
5.2 A	spproach	62
5.2.1	Microfluidic Ratchet Mechanism	62
5.2.2	Cell Separation Using Microfluidic Ratchets	63
5.2.3	Principles of Continuous Filtration Using Microfluidic Ratchet	64
5.3 D	Design of the Microfluidic Ratchet Device	65
5.3.1	Tapered Constriction Design and Modeling	65
5.3.2	Continuous-Flow Microfluidic Device Design for CTC Separation	67
5.4 E	experimental Section	69
5.4.1	Microfluidic Device Fabrication	69
5.4.2	PDMS Device Fabrication	70
5.4.3	Tumor Cell Doping Experiments	71
5.4.4	Microfluidic Device Operation	72
5.5 S	ystem Characterization	73
5.5.1	Device Performance	73
5.5.2	Impact of Deformability on Enrichment of Cancer Cells	75
5.5.3	Viability and Proliferative Capacity of the Enriched Cells	77
		Х

5.6 C	Optimization of CTC Separation for Higher Throughput	
5.6.1	Red Blood Cell Lysis Process Selection	
5.6.2	Multiplexed Inlet Microfluidic Device Design and Operation	80
5.6.3	Multiplexed Inlet Device Performance using Lysed Blood Sample	81
5.7 C	CTC Identification Process Development	
5.7.1	Immunofluorescences Staining for CTC Identification	
5.7.2	Spectral Image Analysis	
5.8 E	Discussion and Summary	
Chapter 6	Development of Single Cell Isolation and Analysis Workflow	92
6.1 II	ntroduction	
6.2 A	Approach	
6.3 E	Experimental Section	
6.3.1	Cell Lines and Sample Preparation	
6.3.2	Identification of Tumor Cells using Fixation-Free Immunofluorescence	
6.3.3	Cell Encapsulation using a Hydrogel Matrix	
6.3.4	Single Cell Extraction Using LCM	
6.3.5	Whole Genome Amplification (WGA) of Extracted Single Cells	
6.3.6	Sanger Sequencing	
6.3.7	Targeted Next Generation Sequencing	
6.4 D	Development of Single-Cell Isolation and Analysis Workflow	100
6.4.1	Fixation-Free Staining and DNA Quality Check	100
6.4.2	Cell Encapsulation Using a Hydrogel matrix	102
6.4.3	Single Cell Extraction using Laser Capture Microdissection	103
		xi

6.4.4 CTC Enumeration using a Customized Software
6.5 Validation for Single-Cell Analysis Workflow 107
6.5.1 Demonstration of Pure Tumor Cells Separations from a Mixed Population
6.5.2 Validation of Single-cell Analysis Workflow using Spiked Sample 108
6.5.3 Validation of Single-cell Analysis Workflow using Next Generation Sequencing 111
6.6 Discussion and Summary 113
Chapter 7: CTC separation, identification and single CTC sequencing in prostate
cancer115
7.1 Introduction
7.2 Overall Approach
7.3 CTC Separation from Patients with Castration-Resistant Prostate Cancer 117
7.3.1 Experimental Section
7.3.1.1 Patient Sample Preparation and Processing
7.3.1.2 CTC Identification
7.3.1.3 CellSearch Analysis 119
7.3.2 Results and Discussion
7.4 CTC Separation from Patients with Localized Prostate Cancer
7.4.1 Experimental Section
7.4.1.1 Patient Sample Preparation and Processing
7.4.1.2 CTC Enrichments from Patients with Localized Prostate Cancer
7.4.1.3 Statistical Analysis
7.4.2 Results and Discussion

7.5	Single-Cell Sequencing of CTCs from Patients with Castration-Resistance	Prostate
Cancer		129
7.5.1	Experimental Section	129
7.5	5.1.1 Patient Blood Sample Collection	129
7.5	5.1.2 Red Blood Cell Lysis	130
7.5	5.1.3 Microfluidic Ratchet-Based Cell Sorting	130
7.5	5.1.4 CTC Identification and Encapsulation using a Hydrogel matrix	131
7.5	5.1.5 Single CTC Extraction and Sequencing	131
7.5.2	Results and Discussion	132
7.6	Summary	137
Chapter 8	8: Conclusions and Future Work	138
8.1	Summary of Results	138
8.2	Future Work	139
Bibliogra	phy	141
Appendic	es	167
Append	lix A Supplementary Information for Morphological Study of CTCs in Pro	state Cancer
A.1	Patients Information Summary	
Append	lix B Red Blood Cell Lysis Protocols	168
B.1	EasySep Kit for CD45 and GlyA Depletion with HetaSep	168
B.2	Density Gradient Centrifugation using Histopaque 1119	168
B.3	RosetteSep Kit for CD45 Depletion with Histopaque 1077	169
B.4	Red Blood Cell Lysis Buffer (Ammonium Chloride)	
		xiii

B.5	Red Blood Cell Lysis Buffer (gBioscience)	169
Appendix	x C List of Reagents	171
C.1	Antibodies used in Immunofluorescence Staining	171
C.2	Common Reagents used in Immunofluorescence Staining	171

List of Tables

Table 5.1 Comparison among various RBC lysis methods.	79
Table 7.1 Patient characteristics in whole cohort, CTC positive and CTC negative patients	127
Table 7.2 Correlation of CTC counts with pathologic risk parameters.	127
Table A.8.1 Patients information summary	167

List of Figures

Figure 2.1 Metastasis and circulating tumor cells (CTCs). Tumor cells may enter the bloodstream via biological events such as EMT. Once at a distant site, tumor cells may Figure 2.2 Probabilities of overall survival of CRPC patients with <5 and ≥ 5 CTCs in 7.5 ml of peripheral blood before initiation of therapy. Patients with \geq 5 CTCs showed reduced overall survival compared to patients with <5 CTC, with a median overall survival of 11.5 (95% CI, 9.1-13.7 months) and 21.7 (95% CI, 21.3 months to Not Reached) respectively...... 10 Figure 3.1Antibody-coated microposts-based enrichment microfluidic devices. (A) CTC capture on EpCAM-coated microposts in CTC chip and (B) GEDI microfluidic device design. The CTCchip consisted of an array of 78,000 microposts chemically functionalized with the anti-EpCAM antibody to capture EpCAM+ CTCs. The GEDI chip combines positive enrichment using antibody-coated microposts with hydrodynamic chromatography to minimize non-specific Figure 3.2 Microfluidic devices for capturing CTCs using functionalized surfaces: (A) The herringbone chip and (B) Geometrically enhanced mixing chip. Both of these devices enhance cell-antibody collision events by inducing local mixing using structures within the Figure 3.3 (A) The IsoFlux CTC capture system. Samples passes through an isolation zone where magnetically labeled cells are captured on the top of the channel in the presence of a magnet. After processing, the cells are retrieved inside the instrument by lifting the cap off the channel and securing it to either microfuge tube or holder to remove the target cells through pipetting (not shown). (B) The LiquidBiopsy system captures CTCs by labeling of whole blood

with a functionalized ferrofluid followed by high throughput partitioning of CTC from blood Figure 3.4 Microfiltration for CTC separation. (A) CTCs captured using ISET and (B) ScreenCell system. The ISET filters fixed blood samples through 8-um pores in a plastic membrane. The ScreenCell system uses similar plastic filters with a hydrophilic surface and Figure 3.5 Microfluidic microfiltration. (A) 3D membrane microfilter device and (B) Parsortix System. The 3D membrane microfilter device conscists of two porous parylene-C layers. The bottom layer contains hexagonally-arranged 8-µm pores. The top layer contains larger 40-µm pores that align with the corresponding hexagon patterns on the bottom membrane. The Parsortix microdevice has a stair-like structure that gradually decreases the channel width to <10 µm. 27 Figure 3.6 CTC-iChip: (A) Device design and (B) integrated microfluidic system. The microfluidic CTC-iChip system first sorts the various cells in a blood sample by size, allowing only CTCs and white blood cells to enter the inertial focusing chamber, which lines up those cells into a single file. A magnetic field then deflects cells previously labeled with tiny magnetic Figure 3.7 Vortex Chip. (A, B) Design and (C, D, E) cell separation using Vortex Chip. The Vortex chip used for cell trapping consists of 8 channels in parallel with 8 reservoirs. At the channel inlet, cells are randomly distributed and experience two opposing lift forces, the wall effect and the shear-gradient lift force. As a result, particles migrate to dynamic lateral equilibrium positions, depending on the channel cross section. Upon entrance in to the reservoir, the wall effect is reduced. Larger cells (violet dashed line) still experience a large lift force and are pushed away from the channel center through the separatrix and into the vortices, where they are stably trapped. Smaller cells (red dashed line) do not experience enough lift force to cross the separatrix and remain in the main Figure 3.8 ClearCell system. (A) Illustration of the design of a multiplexed device, (B, C) optical image of an actual multiplexed spiral microfluidic device for capturing CTCs with two inlets and two outlets. Blood sample and sheath fluid are pumped through the device using two separate syringe pumps. Under the influence of inertial lift and Dean drag forces in the fluid flow, CTCs focus near microchannel inner wall (Region A-A) while WBCs and Platelets goes through one Dean cycle and migrate back towards the outer wall (Region B-B), thus achieving separation...31 Figure 3.9 Schematic representations of ApoStream device. (A) ApoStream prototype instrument, (B) 3D CAD model of the flow chamber, and (C) DEP enrichment for CTCs. The sample injection port introduces cells at the bottom of the flow chamber upstream of the buffer. Electrodes line the bottom of the flow chamber and generate an electric filed that attracts CTCs Figure 3.11 NanoVelcro chip. (A) The configuration of PLGA nanofiber (PN)-NanoVelcro Chip, (B) a SEM image of the electrospun PLGA nanofibers, and (C) schematic illustration of the Figure 4.1 A screen shot of image analysis software developed by Richard R. Ang using LabView. The program acquires the images for each CTC candidate. A selected image (highlighted in yellow) is analyzed to measure the area in pixels. An ellipse is fitted to this image and overlaid on top of the original image for checking. Parameters for intermediate image

Figure 4.2 Data processing algorithm for the image analysis software developed by Richard R.
Ang. Images acquired from the CellSearch® are analyzed via to determine (A) the overall area,
and (B) the best-fit ellipse
Figure 4.3 Rejected cells from CTC identification. These images of cell fragments commonly
appeared during image analysis and were not included in the CTC count or cell size
measurements. Typical CTC fragments include a nucleus partly covered by cytokeratin, or a
nucleus completely separated from cytokeratin. These fragments likely originated from CTCs
undergoing apoptosis. The yellow scale bar is 5 µm in length
Figure 4.4 Diameters of CTCs from prostate cancer patients (pre-treatment) and cultured prostate
cancer cells. The average diameter of CTCs (7.97 μ m) was significantly smaller than cultured
cancer cells (13.38 µm) (p<0.001)
Figure 4.5 Correlation between cell size and CTCs count. There appeared to be no correlation
between CTC cell size and cell count for CTCs identified by CellSearch from patients with
metastatic castrate resistant prostate cancer. The cell size ranged from 6.9 μ m to 8.95 μ m; while
the CTC count varied from 11 to 106
Figure 4.6 Example images of cultured prostate cancer cell (A–D) and CTCs from prostate
cancer patients (E-L) captured using the CellSearch® system. CTCs were noticeably smaller than
cultured cancer cells (A–D). Cultured cancer cells were mostly round with regular cell and
nuclear shapes. The nucleus was typically centered and surrounded by cytokeratin (E-L). CTCs
exhibited highly variable shapes, including round (E), oval (F), elongated (G–J), and clusters (L).
Non-round and multi-nucleate cells were sometimes observed (G-K). The yellow scale bar is 5
μm in length

Figure 4.7 (A) Elongation factor (EF) of CTCs from prostate cancer patients (in blue) compared with cultured prostate cancer cells (in grey). The median EF of CTCs was generally greater with significant inter- and intra-patient variability. (B) Nuclear cytoplasmic (N/C) ratios of CTCs from prostate cancer patients (in blue) compared with cultured prostate cancer cells (in grey). Median with upper and lower quartiles is shown for each sample. The median N/C ratio for Figure 4.8 Changes in cell and nucleus after two days of storage in the CellSave® tubes. The diameter of cultured prostate cancer cells decreased $\sim 6\%$ on average while the nuclear diameter Figure 5.1 Principle of microfluidic ratchet mechanism. Larger and less deformable cells are prevented from transiting through funnel constitutions in forward flow (indicated by red arrow, A). Smaller and more deformable cells are unable to return through funnel constrictions under Figure 5.2 Continuous deformability-based cell separation using oscillatory diagonal flow through a matrix of funnel constrictions. The blood sample infused into the bottom-left of the funnel matrix travels in a diagonal path until reaching a limiting funnel size. The path of the less deformable CTCs (shown in purple) flattens at a larger funnel row than the more deformable Figure 5.3 (A) Structure of the tapered constriction determined by the parameters of microchannel thickness (H_0) , pore size (W_0) , and funnel taper shape (f(x)). (B,C) Modeling single cell as liquid drop to estimate its deformation pressure along and against direction of taper.

Figure 5.4 (A) Photograph of the microfluidic ratchet device infused with colored water to show the diagonal flow pattern in the separation matrix. (B) Composite fluorescence image of leukocytes (blue) and doped UM-UC13 cancer cells (green) in the funnel matrix during a Figure 5.5 (A,B) Performance of deformability-based separation of UM-UC13 cancer cells doped into whole blood at the cancer cell to leukocyte ratio of 1000:1. Enrichment and yield are characterized as a function of deformation pressure (at the 6 µm cutoff funnels). (C,D) Relationship of doping ratio and deformability-based system performance. Enrichment and yield Figure 5.6 (A) Size distribution of leukocytes and UM-UC13 cancer cells. The black lines represent fitted normal distribution for the size measurements. B) Enrichment of doped UM-UC13 cancer cells obtained through deformability based separation using the microfluidic ratchet device compared to the optimal performance obtained through size-only separation calculated from cell size distribution in A......76 Figure 5.7 (A) Cell growth of UM-UC13 cells after cell separation. (B) Cell number increase during subsequent culture. (C) Assessment of cell metabolic activity based on MTT assay...... 77 Figure 5.8 (A) Representative image of the device with a multiplex sample inlet. (B) Photograph Figure 5.9 (A) Yield comparison between the continuous-flow device (version 2.0) and the multiplexed inlet microfluidic device (version 2.1). (B) Yield comparison between RBC lysis

Figure 5.10 LNCaP simultaneously stained with for CK, AR, EpCAM, CD45 and DAPI. (B)
Leukocytes stained negative for epithelial (EpCAM and CK) and prostate tumor (AR) antigens
but positive for CD45
Figure 5.11 LNCaP identified as DAPI+CK+EpCAM+CD45- and leukocytes identified as
DAPI+CD45+
Figure 5.12 Emission spectra of Alexa 488, Alexa 555, Alexa 594, and APC, demonstrating its
own distinct peak. Solid lines represent the emission wavelengths for each fluorochromes, while
dotted lines represent the excitation wavelengths. [Image generated from Fluorescence
SpectraViewer, ThermoFisher]
Figure 5.13 Fluorescence microscopy image and corresponding spectra for LNCaP
(DAPI+CK+EpCAM+CD45-) and leukocytes (DAPI+CD45+)
Figure 6.1 PDMS baffle. It allows the concentration and deposition of cells on a glass slide using
centrifugation
Figure 6.2 Potential DNA damage on PFA-fixed single cells after WGA. Fixation of specimens
with PFA reduces the efficiency of subsequent WGA. The integrated transgene mCherry and
endogenous 18S rDNA were targeted for quantification by qPCR. Target gene abundance was
consistently lower in DNA isolates from PFA-treated cells, with mCherry loci only robustly
detected in non-fixed UC3-mCherry specimens
Figure 6.3 Comparison of standard immunostaining of LNCaP tumor cells (left image) for CD45
(red), EpCAM (green) and Hoechst (blue), with live staining using the same antibodies. Live
staining of tumor cells showed similar cell specificity and fluorescence intensity as tumor cells
stained using the standard protocol

Figure 6.4 Single cell extraction using LCM. A single UC-13 cell transfected with EGFP is marked and extracted using laser microdissection system. The captured cell is visually confirmed Figure 6.5 Gigapixel Spectral Image Cube. Peak channels are listed as well as the quality control bright field channel which is obtained from the transmitted light through the sample while all color channels are fluorescent emissions from the sample......105 Figure 6.7 (A) Single UC13 cell extraction using LCM. A single UC-13 cell transfected with EGFP is marked and extracted using laser microdissection system. The captured cell is visually confirmed on a cap of a capture tube. (B, C) Two cell lines UM-UC13-EGFP and UM-UC3mCherry were intentionally combined in suspension and four single cells from each cell line, as well as a no-template (NT) and cell line reference DNA (gDNA) controls, were subjected to WGA and analyzed by transgene-specific qPCR. The black arrows indicate where qPCR failed to amplify the target DNA locus Successful extraction of target cells was indicated by detection of that cell's specific transgene but not the transgene of the contaminant cell line...... 107 Figure 6.8 (A) Single-cell analysis workflow. (B) Target cells are identified using our customized software, and the positional information was stored as a CTC map. The CTC map was used to identify target cells for single cell extraction using LCM. (C) Following WGA, we successfully amplified the AR exon 8 region by PCR from single LNCaP (L1-L3) and single 22RV1 (R1 and R2) cells, as well as CD45+ leukocytes within the enriched specimen (W1 and W2). An unoccupied segment of the PEGDA membrane was excised as a negative control (P). (D) Sanger sequencing of the PCR products revealed the previously established T877A and

H874Y mutations in LNCaP and 22RV1, respectively, while the leukocyte amplicons (WBC) Figure 6.9 Scatter plots demonstrating the concordance between each pair of samples for mutations or single nucleotide polymorphism (SNP) present at >5% frequency in UC13 and UC3 Figure 7.1 (A,B) Fluorescence microscopy image and corresponding spectra for CTCs (DAPI+CK+CD45-) and leukocytes (DAPI+CK-CD45+). (C,D) CTC enumeration comparison between the microfluidic ratchet device and the CellSearch[®] system. The p-value indicates a significant difference by Wilcoxon-ranked sum. (E) Comparison of cell size between leukocytes Figure 7.2 Immunofluorescence images and spectral analysis for CTCs (DAPI+CK+CD45-) and Figure 7.3 (A) CTCs (DAPI+/EpCAM+/CD45-), enriched from blood of patients with castration resistant prostate cancer, were imaged alongside contaminant leukocytes (WBCs; DAPI+/EpCAM-/CD45+). (B) Spectral analysis discriminated CTCs based on the CTC-specific peak at 520 nm (EpCAM-Alexa488) and the WBC-specific peak 660 nm (CD45-APC). (C) Image illustrating the extraction of a single CTC using the LCM system, as well as the confirmed collection of this cell on a cap of the collection tube. (D) Following single cell extraction and WGA, a qPCR was performed to amplify the ACTB locus as a confirmation of template quality. Figure 7.4 Next generation sequencing was performed to compare cell free DNA (cfDNA) with single CTCs and a single leukocyte (WBC). A) Forty five somatic mutations were detected that xxiv

List of Abbreviations

AR	Androgen Receptor
BSA	Bovine Serum Albumin
cfDNA	Cell-Free DNA
СК	Cytokeratin
CRPC	Castration-Resistant Prostate Cancer
CTC	Circulating Tumor Cells
DEP	Dielectrophoresis
DLD	Deterministic Lateral Displacement
EF	Elongation Factor
EMT	Epithelial–Mesenchymal Transition
EpCAM	Epithelial Cellular Adhesion Molecule
FACS	Fluorescence-activated Cell Sorting
FDA	Food and Drug Administration
FISH	Fluorescence in situ Hybridization
IR	Infrared
LCM	Laser Capture Microdissection
MET	Mesenchymal-to-Epithelial Transition
N/C	Nuclear to Cytoplasmic
NGS	Next Generation Sequencing
PDMS	Polydimethylsiloxane
PBS	Phosphate Buffered Saline

PEN	Polyethylene Naphthalate
PNG	Portable Network Graphics
PSA	Prostate-specific Antigen
PSMA	Prostate-specific Membrane Antigen
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
UV	Ultraviolet
WBC	White Blood Cell
WGA	Whole Genome Amplification

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Hongshen Ma for his patient guidance, encouragement and advice he has provided throughout my PhD study. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my PhD study. I also would like to thank all of the members in the Multi-Scale Design Laboratory for their support. Many thanks to Chao Jin, Justin Yan, Richard Ang, and Xiaoyan Deng for the stimulating discussions and for all the fun we have had together. Special thanks go to Dr. Simon Duffy for his constructive suggestions he made to this thesis and many other manuscripts we worked together.

I also want to give my thanks to staffs and colleagues in Vancouver Prostate Centre for their assistance. My sincere thanks go to Dr. Peter Black, who recruited patient samples and gave access to the laboratory and research facilities. Without his precious support it would not be possible to conduct this research. I would also like to acknowledge the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre for their collaboration in the genome sequencing studies.

Last but not the least, I would like to thank my family: my husband, my parents and my brother for their love and support during my PhD study. My warmest thanks to my beloved daughters for their smiles that always cheer me up.

Dedication

This thesis work is dedicated to my husband, Youngil Choi, who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in my life.

This work is also dedicated to my parents, Jongsam Park and Kisook Lee, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

Lastly, this work is dedicated to my daughters, Seora and Hyobi. You have no idea of the amount of happiness you bought into my life.

Chapter 1: Introduction

1.1 Significance and Motivation

Circulating tumor cells (CTCs) are malignant cells shed from a primary tumor into the bloodstream, where they have the potential to form metastases that are ultimately responsible for >90% of all cancer related deaths^{1,2}. Since CTCs represent the critical transitional state between primary and metastatic tumors, the enumeration and characterization of CTCs has the potential to be a minimally invasive and longitudinal method to evaluate cancer disease status and treatment efficacy^{3,4}. CTCs are particularly important for prostate cancer because the site of metastasis is predominantly in the bone, making biopsy difficult and low yield⁵. CTCs can be obtained from blood to provide rapid, non-invasive, and longitudinal access to key tumor cells relevant to metastasis. In fact, many research groups are currently studying the value of biological information obtained from CTCs as a prognostic marker for estimating overall survival^{6–9}, a diagnostic marker for identifying the disease status^{10–12}, a predictive marker for personalizing the therapy^{9,13,14}, and a surrogate marker for developing new drugs¹⁵.

A key challenge in the separation and identification of CTCs from patient blood is their extreme rarity, where as few as one CTC in $\sim 5 \times 10^6$ leukocytes has been reported^{16,17}. CTC enumeration methods typically overcome this needle-in-a-haystack problem using an initial enrichment step followed by immunofluorescence to positively identify the CTCs^{18–20}. While there has been a wide array of CTC enrichment methods developed, the strategies can be broadly divided into two categories, affinity-based methods and biophysical methods, each with distinct strengths and limitations. The key strength of affinity-based enrichment methods is that they are highly selective because they typically capture cells expressing the epithelial-specific EpCAM antigen,

which is not expressed by normal blood cells^{21–24}. However, this advantage of affinity based enrichment is also its primary limitation because a key step in tumor invasion and metastasis is epithelial-mesenchymal transition (EMT), a phenotypic switch where expression of EpCAM and other epithelial biomarkers may be diminished^{25–27}. Consequently, the aggressively metastatic CTCs are the most likely group to be missed by methods that rely on EpCAM immunoaffinity capture. This antigen bias has promoted the development of biophysical 'label-free' CTC enrichment methods. These methods typically employ size-based separation using microfiltration, inertial focusing, or dielectrophoresis and have successfully detected CTCs in clinical samples. However, these methods may have limited selectivity for CTCs, owing to size overlap between CTCs and leukocytes, which contributes to the loss of CTCs and retention of contaminant leukocytes. Consequently, there is a significant need to develop improved technologies for CTC separation.

After separating CTCs from patient blood, a longstanding technical challenge is to sequence the genomes of individual CTCs. Currently, there are two key barriers to achieving this goal: (1) CTC separation technologies invariably produce an impure sample with contaminant leukocytes and erythrocytes, and (2) CTCs are also a heterogeneous group with significant cell-to-cell variability²⁸. Recent advances in whole genome amplification have enabled the generation of sequencing libraries from single cells with >90% coverage²⁹, however, key mutations/alleles could be easily masked by contaminant leukocytes or CTC heterogeneity. Therefore, there is a tremendous need to develop methods to enrich CTCs to high purity and then to isolate and sequence individual CTCs. Single CTC sequencing performed in published studies have been obtained through micropipette aspiration of enriched CTCs^{30–33}. This approach, however, is

technically challenging and time-consuming^{30–32,34}, and cannot be scaled-up to perform hypothesis testing in clinical trials where significant patient numbers are required to overcome disease and patient heterogeneity.

1.2 Research Objectives

This dissertation describes research performed to 1) analyze the morphology of CTCs, 2) develop a technology to enrich for CTCs and an analytical pipeline to rapidly identify CTCs, 3) develop a workflow to extract single CTCs for genome sequencing, as well as 4) the application of these technologies to study patients with metastatic castration-resistant prostate cancer (CRPC) and localized prostate cancer.

1.3 Organization of the Thesis

Chapter 2 reviews current knowledge on the biology of CTCs, as well as recent studies of CTCs in advanced and localized prostate cancer. Chapter 3 reviews recent work in CTC separation technologies, single cell isolation technologies, and recent work in single CTC sequencing. Chapter 4 presents the morphology study in CTCs from patients with metastatic prostate cancer enriched by CellSearch[®] system. Specifically, we developed a software to analyze the images of patient-driven CTCs from CellSearch[®] system in order to investigate the cytomophological properties of cancer cells including eccentricity of cell shape, size and nuclear cytoplasmic ratio. Chapter 5 introduces the microfluidic ratchet mechanism and describes the development and validation of a microfluidic device to separate CTCs and analytical pipeline. Chapter 6 presents the development and validation of the single cell analysis workflow using laser capture microdiseection (LCM) system, including single cell extraction, whole genome amplification,

and genome sequencing. Chapter 7 presents the application of the CTC separation and detection technology to enumerate CTCs from patients with metastatic and localized prostate cancer, as well as single CTC sequencing from a single patient with metastatic prostate cancer. Finally, Chapter 8 presents the conclusions and potential future directions arising from this dissertation.

Chapter 2: Background

This chapter reviews the biology of circulating tumor cells (CTCs), as well as recent CTC research in prostate cancer, including metastatic castration-resistant prostate cancer (CPRC) and localized prostate cancer.

2.1 Metastasis and Circulating Tumor Cells (CTCs)

Metastasis is the spread of tumors from its primary site to the anatomically distant organs. This process is ultimately responsible for over 90% of all cancer related deaths^{1,2}. Under the seed-and-soil hypothesis, metastasis is a product of favorable interaction with metastatic tumor cells (the "seed") and microenvironment of select organs (the "soil")³⁵. Thus, metastases occur only when the appropriate seed was implanted in suitable soil. The process of metastasis consists of a series of steps that begins when tumor cells detach from the primary tumor to enter the bloodstream via biological changes such as epithelial to mesenchymal transition (EMT)^{36,37}. In order to enter the bloodstream, tumor cells lose their polarities and adhesive bonds with neighboring tumor



Figure 2.1 Metastasis and circulating tumor cells (CTCs). Tumor cells may enter the bloodstream via biological events such as EMT. Once at a distant site, tumor cells may extravasate, undergo MET, and grow locally to become a metastasis.

[reproduced from Joosse et al.³⁷ under CC BY 4.0]

cells and gain the ability to penetrate basement membranes^{38–40}. Typical epithelial markers including EpCAM and E-cadherin are down-regulated whereas up-regulation of mesenchymal markers such as vimentin is observed on tumor cells during EMT^{41–43}. Those tumor cells mediating metastasis are called circulating tumor cells (CTCs). Once at an anatomically distant site, CTCs undergo reverse mesenchymal-to-epithelial transition (MET) to acquire the ability to implant and form a metastatic tumor^{37,44}.

CTCs was first reported nearly 150 years ago by Thomas Ashworth who noticed some unusual cells in the peripheral blood of a patient with metastatic cancer⁴⁵. He observed that many morphologic features were shared between the cells found in the solid tumors from different lesions and these circulating cells, and concluded "One thing is certain, that if they [CTC] came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg".⁴⁵ Since this early observation, many research groups have been proposed CTC enrichment and characterization method to study these cells and cancer biology, aiming for exploring CTCs as tumor biomarkers. In clinical trials, CTC counts that are enumerated using the CellSearch® platform (Veridex, Raritan, NJ) have been shown to correlate with overall survival^{7,8,20,24,46-50}, where the presence of \geq 5 CTCs in 7.5 mL of whole blood from breast and prostate cancer, and \geq 3 CTCs in colorectal cancer has been shown to be predictive of unfavorable prognosis^{46,48,50}. Beyond predicting outcomes, changes in CTC count during anticancer therapy can also indicate sensitivity or resistance to the therapy. Patients with a significant decrease in CTC number after treatment often show better outcomes 50-52. In metastatic colorectal cancer, the median overall

survival for patients who began with unfavorable CTCs (\geq 5 CTC/7.5 mL enriched by CellSearch[®]) but converted to the favorable group after the treatment was significantly longer compared with that of patients who remained with unfavorable CTCs at both time points (11.0 versus 3.7 months)⁵². Furthermore, some studies have suggested that enumeration of CTCs could serve as a surrogate measure of clinical outcomes^{15,53,54}. For example, CTC enumeration in docetaxel-treated patients with castration-resistant prostate cancer (CRPC) treated with abiraterone acetate met surrogacy endpoints in trials¹⁵. After treatment with abiraterone acetate, CTC counts were declined in 41% of patients, and those results were supported by prostate specific antigen (PSA) decline, radiologic responses, and improvement in symptoms.

Despite the potential value of CTCs, there are two critical challenges associated with extracting useful information from CTCs. First, CTCs are extremely rare in blood^{16,17} (1-10 CTCs per million normal blood cells). At this rarity, isolates are typically contaminated by leukocytes, leading to the need to develop highly efficient and sensitive CTC enrichment methods. Second, CTCs are heterogeneous, which creates a need to characterize CTCs individually. For example, tumor cells from different parts of the same tumor showed different genomic profiles⁵⁵. In breast cancer, tumor cells collected within the same tumor were either wild-type or contains PIK3CA mutations⁵⁶. This reflects significant intratumor diversity, and thus CTCs from different parts of tumors more accurately represent existing heterogeneity than could be revealed by a single tissue biopsy. Furthermore, CTCs may undergo the epithelial-to-mesenchymal transition (EMT), where tumor cells undergo a phenotypic switch that is accompanied by a loss of epithelial biomarkers (e.g. EpCAM) and a gain of mesenchymal markers (e.g. vimentin)⁵⁷. EMT progress in individual tumor cells can be varying depending on cells' invasive potentials, leading to significant
heterogeneity among CTCs. Wu et al. demonstrated that mesenchymal CTCs were more commonly found in patients with metastatic stages of disease, compared with the earlier stages of cancer⁵⁸. This heterogeneity characteristics of CTCs highlights the importance of analyzing CTCs at the single-cell level because key mutations from individual cells can be easily lost in bulk analysis^{59,60}. Thus, beyond CTC enumeration, genomic profiling of single CTCs can help us better understand CTCs, providing great insights into evolution and mechanism of cancer metastasis. The improved understanding of metastasis will ultimately help clinicians for monitoring disease progression and therapeutic decision making.

2.2 CTCs in Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer in North American men and is the second most common cause of cancer deaths^{61,62}. Approximately 85% of newly diagnosed prostate cancers are localized to the prostate, with the remainder showing dissemination to other tissues and representing advanced or metastatic disease⁶³. Advanced prostate cancer is typically classified into four subsets⁶⁴: (1) locally advanced prostate cancer where the tumor has started to invade tissues immediately surrounding the prostate, (2) metastatic prostate cancer where the tumor has spread beyond the prostate, most commonly to lymph glands in the pelvic region and bones, (3) recurrent prostate cancer where the cancer that returns after initial therapy such as radiation therapy or surgery, and (4) metastatic castration-resistant prostate cancer (CRPC) where the disease has already been treated with hormonal therapy, but has developed resistance. Locally advanced, metastatic, and recurrent prostate cancers are currently treated with androgen deprivation, which prolongs overall survival, but the disease ultimately progresses to 'androgen-independent' or 'CRPC⁶³.

Metastatic prostate cancers carry the greatest risk for mortality because tumor cells can disseminate systemically through the bloodstream and deposit within tissues that are not easily accessible for biopsy. For example, prostate tumors frequently initiate metastases in bone marrow⁶⁵, which has been found to represents 80% of cases of metastatic disease⁶⁶. Tissue biopsy of bone marrow is highly invasive and often technically challenging⁶⁷, making it infeasible to perform routinely (e.g. monthly or after each treatment). A compelling alternative to tissue biopsy is the direct capture of circulating tumor cells (CTCs) from the peripheral bloodstream. CTCs are uniquely relevant to the characterization of metastasis because this cell population potentially includes tumor cell sloughed from the localized primary tumor and the metastatic tumor, as well as potential circulating metastasis-inducing cells. Since some CTCs share genetic characteristics of the metastatic tumor, they represent a non-invasive alternative to tissue biopsy that can be used for longitudinal monitoring of therapeutic efficacy. Furthermore, the recent detection of CTCs in prostate cancer patients without distant metastases^{68–70} suggests that these cells may serve as a biomarker to identify and stratify patients with early stage cancer, providing these patients with upfront treatment.

2.2.1 CTCs in Castration-Resistant Prostate Cancer (CRPC)

To date, CTCs have been most extensively studied as a prognostic biomarker in metastatic prostate cancer. Several studies reported a strong correlation between CTC counts and clinical outcomes in patients with metastasis^{6,49,54}. For example, de Bono et al. studied CTC counts in 231 patients with CRPC before and after chemotherapy, and reported that CRPC patients with unfavorable CTC (\geq 5 CTC/7.5 mL enriched by CellSearch[®]) were correlated with reduced overall survival compared to patients with favorable CTC (<5 CTCs/7.5 mL) (11.5 vs. 21.7



Figure 2.2 Probabilities of overall survival of CRPC patients with <5 and ≥5 CTCs in 7.5 ml of peripheral blood before initiation of therapy. Patients with ≥5 CTCs showed reduced overall survival compared to patients with <5 CTC, with a median overall survival of 11.5 (95% CI, 9.1-13.7 months) and 21.7 (95% CI, 21.3 months to Not Reached) respectively.

[reproduced from de Bono et al.⁶ with permission from American Association for Cancer Research]

months, p < 0.0001) as shown in Figure 2.2. In addition, decline in CTC counts after treatment was significantly associated with an overall survival rate⁶. Patients with unfavorable baseline CTC (\geq 5CTCs/7.5mL) who converted to favorable CTC (<5CTCs/7.5mL) had improved overall survival (6.5 to 21.3 months; p<0.0001) after the treatment. Conversely, patients who had a change from favorable (<5) to unfavorable (\geq 5) CTC count had a significantly worse survival when compared to those with continuously favorable CTC counts (>26 vs. 9.3 months; p<0.0001).

In addition to CTC enumeration, molecular analysis of CTCs from patients with CRPC offers insight into biological status of the tumor, providing great promise for evaluating disease status

and drug efficacy. The androgen receptor (AR) plays an important role in prostate tumor progression, and thus AR has been extensively studied in CTCs from CRPC patients. High-level AR amplification in CellSearch-enriched CTCs was observed in 35% of CRPC patients (17/49) by fluorescent in situ hybridization (FISH), wherein 50 % of the patients had ≥ 10 CTC/7.5mL blood⁷¹. Another study utilized single cell immunofluorescence assay to measure changes in AR activity within CTCs, enriched by an EpCAM-based microfluidic device⁷². In this study, CTCs were identified based on prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) expression, which categorized CTCs in three different phenotypes: AR-on (PSA+/PMSA-), AR-mixed (PSA+/PSMA+), and AR-off (PSA-/PSMA+). AR activity was predominantly positive amongst the patients with newly diagnosed metastatic prostate cancer prior to the initiation of androgen deprivation therapy. Vast majority of CTCs from these patients showed the "AR-on" (PSA+/PSMA-) phenotype (99.1%), and the androgen deprivation therapy resulted in a transformation from AR-on to AR-off signature. In contrast, CRPC patients with detectable CTCs (14/20; 70%) demonstrated a significant heterogeneity in CTC AR activity. CTCs from patients with CRPC fall into AR-off (median 51.95%) and AR-mixed (median 17.6%). In response to the treatment (e.g. abiraterone acetate which blocks androgen synthesis), 24% (4/17) of CRPC patients had a 50% or more decline in the percentage of AR-on CTCs within 2 to 5 weeks of therapy. In addition, an increase in the percentage of AR-on CTCs despite abiraterone therapy was correlated with decreased overall survival. These results highlight the potential importance of CTCs as dynamic or 'real-time' biomarkers that can reflect the drug efficacy and the disease status in response to the treatment.

Recent progress on CTC separation and characterization techniques allowed the genomic profiling of CTCs from CRPC patients at single-cell level. This single-cell analysis has demonstrated that there is substantial genetic heterogeneity within the CTC population, but that these genetic variants were shared with the originating tumor tissue. These studies therefore support the use of CTC analysis as an alternative to tissue biopsy by providing a representative sampling of the patient tumor. Recently, Jiang et al. reported the molecular similarity between single CTCs (n=12) and tissues from primary and metastatic tumors isolated from a patient with metastatic prostate cancer at the single cell level using whole genome sequencing⁷³. In this study, 86% of the clonal mutations identified in CTCs could be traced back to either the primary or metastatic tumors, supporting the argument that CTC populations are representative of the originating tumor. In addition, they found highly heterogeneous short variations in chromosome structure (e.g. inversion, balanced translocation, etc.) were discovered in PTEN, RB1, and BRCA2 in all tumor and CTC samples in prostate cancer. Lohr and colleagues performed wholeexome sequencing on single CTCs as well as metastatic and primary tumor tissues. High portion of CTC mutations (70%) were present in matched tissue, confirming that CTCs were genetically related to the primary prostate cancer. These results suggested that analysis of CTCs can be very useful when the metastatic tissue is not accessible because CTCs which share genetic characteristics with primary or metastatic tumors. This is particularly important in prostate cancer, where the site of metastasis is predominantly in the bone. Beyond the genomic profiling, Miyamoto et al. recently performed single cell RNA-sequencing (RNA-seq) profiles of 77 intact CTCs isolated from 13 CRPC patients (average of 6 CTCs per patient) using microfluidic enrichment, and found considerable heterogeneity in individual CTCs, including AR gene mutations and splicing variants. This study also showed that AR-targeted drug resistance (e.g.

enzalutamide) was associated with activation of non-canonical Wnt signaling⁷⁴. All of these results from single-cell analysis suggest that molecular characterization of CTCs may be of value for studying the tumor evolution as well as monitoring the drug response in patients with metastatic prostate cancer.

In summary, CTC enumeration and their molecular characterization may be a valuable tool to monitor the disease progression and response to therapy as well as drug selection in patients with CRPC. However, of even greater value is the development of techniques for single-cell genetic characterization of CTCs because these methods may provide a deeper insight into the heterogeneity of CTCs the molecular mechanisms of tumor progression.

2.2.2 CTCs in Localized Prostate Cancer

The detection of CTCs in patients with localized prostate cancer is expected to be more challenging than in patients with metastatic prostate cancer because cells sloughed from the primary tumor would have low persistence in blood. Furthermore, unlike metastatic prostate cancer, the value of CTC detection in localized prostate cancer is also unkown⁷⁵. For instance, Davis *et al.* detected CTCs in 21 % (20/97) of patients with localized prostate cancer using CellSearch[®] but similar proportion (20%) of the control group was also positive for CTC⁶⁹. Among the 20 patients with cancer positive for CTCs at baseline, 18 had no detectable CTCs after radical prostatectomy, CTCs were found at very low levels for two men CTCs (1 CTC/22.5mL). The limitation of this study is that 22.5 mL of blood was used to detect CTCs in localized patient samples, instead of 7.5 mL of blood, which is usually processed when using the CellSearch[®] system. Considering higher blood volume used in this study, the number of patients

with ≥ 1 CTC should have been much lower if the usual amount (7.5 mL) was processed. Similar results were obtained by Thalgott et al., who analyzed CTCs in 20 patients with localized cancer using CellSearch[®] and detected 1 CTC in 1 patient (5%)⁶⁸. Again, they did not see a difference in positivity rate between patients with localized prostate cancer and healthy controls. Another study by Meyer et al. detected CTCs with a median CTC count of 1/7.5 mL (range:1 to >100) using CellSearch[®] in 17 of 152 patients (11.2%) with localized prostate cancer after radical prostatectomy⁷⁵. There was no significant correlation with the presence of CTCs with other pathologic risk parameters such as T-stage, Gleason score or PSA level. After a median followup of 48 months, there was no significant difference in biochemical recurrence-free survival between patients with or without CTCs. On the other hand, Pal et al. detected CTCs in 49% of patients (17/35) with high risk localized prostate cancer using a modification of the CellSearch[®] protocol⁷⁶. They added a stem cell marker (CD133) and an EMT marker (E-Cadherin) to an open channel in CellSearch[®] platform, increasing the sensitivity of the system for localized samples. Although no correlation between CTC count and biochemical recurrence (BR) free survival was observed, the percentages of CD133 and E-cadherin-positive CTC fragments were correlated with biochemical recurrence one-year following surgery

Overall, unlike the metastatic prostate cancer, the clinical value of CTC analysis in localized prostate cancer remains to be determined. Recent research is beginning to demonstrate the possibility of detecting CTCs in these patients and the potential utility of characterizing these cells as a diagnostic or prognostic tool.

Chapter 3: Literature Review

This chapter reviews recent research in CTC separation, single cell isolation, and single CTC sequencing. First, two major categories of CTC separation methods, affinity-based and biophysical property-based methods, are discussed. Next, single cell isolation platforms including fluorescence activated cell sorting (FACS), micromanipulation, and laser capture microdissection (LCM) are reviewed. Lastly, single CTC sequencing studies for various cancer types will be reviewed.

3.1 CTC Separation Methods

Current methods for CTC separation are classified under two major categories: (1) affinity-based and (2) biophysical-based cell sorting. The first section will focus on affinity-based methods, which utilize unique antigen(s) expression on CTCs. The second section will focus on biophysical-based cell sorting, which utilize distinct biophysical characteristics of cancer cells, including size, deformability and/or electrical charge to enrich for cancer cells and deplete hematological cells. Several examples of each approach will be presented along with a discussion of their advantages and disadvantage.

3.1.1 Affinity-based Enrichment

Affinity-based enrichment can be classified into positive selection methods and negative selection methods. Positive selection utilizes differences in cell surface antigen between epithelial cells that originated from solid tumors and hematological cells found in the bloodstream. Previous methods in positive selection relies primarily on Epithelial Cell Adhesion Molecule (EpCAM) that is a transmembrane glycoprotein expressed in epithelial tissue⁷⁷. Thus,

antibodies against EpCAM can be used selectively capture epithelial CTCs from whole blood. On the other hand, a negative selection depletes major contaminants blood cells (e.g. leukocytes) using leukocytes common antigen, CD45. Here, several types of affinity-based positive and negative enrichment methods are reviewed. In positive selection methods, commercially available immunomagnetic enrichment systems, CellSearch[®] and MagSweeper, are discussed. Next, various microfluidic devices are discussed in three different categories: (1) micropostsbased enrichment devices such as CTC chip and geometrically enhanced differential immunocapture (GEDI) chip, (2) surface-based enrichment devices such as the herringbone (HB) chip and geometrically enhanced mixing (GEM) chip, and (3) immunomagnetic-based devices such as IsoFlux and LiquidBiopsy. Lastly, negative depletion systems, including EasySep system and microfluidic CTC-iChip will be discussed.

3.1.1.1 Positive Selection

The majority of positive enrichment technologies for CTCs in various epithelial-origin cancer types (e.g. breast, prostate, and colon) have targeted the epithelial cell surface antigen (e.g. EpCAM), with subsequent immunofluorescence identification using cytokeratin (CK), and nuclear staining (DAPI)⁷⁸. Using this approach, Veridex developed the benchtop device, the CellSearch[®], that is the only FDA-approved technology for clinical CTC testing in metastatic prostate, breast and colorectal cancers^{7,18,47}. The CellSearch[®] platform employs the immunomagnetic capture of CTCs using magnetic nanoparticles that are coupled with anti-EpCAM antibodies to enrich CTCs with EpCAM expression. After enrichment, captured cells are stained for Cytokeratin (CK), CD45, and DAPI to identify CTCs as CK+CD45-DAPI+ and leukocytes as CK-CD45+DAPI+²⁴. In clinical practice, CTC counts (\geq 5 per 7.5 ml of blood

samples for breast⁷ and prostate cancers⁶ or \geq 3 for colorectal cancer⁹) have been associated with poor prognosis. As the CellSearch[®] system is considered as the "golden standard" in CTC enumeration, many newly developed CTC enrichment methods compare their results to CellSearch[®] to validate their system.

Another commercially available system for CTC enumeration based on immunomagnetic separation is the MagSweeper (Illumina Inc.)⁷⁹. This system consists of a robotic arm with a magnetic rod that continuously sweeps through wells containing labeled sample. Using precise control of speed and trajectory of the rod movement, the system is optimized to capture only EpCAM-labeled CTCs, preventing the non-specific adsorption of non-magnetically labeled cells⁷⁹. Thus, the MagSweeper can produce relatively high purity sample after enrichment compared to CellSearch[®], allowing downstream molecular analysis, which can be easily hampered by leukocytes contamination. For example, the MagSweeper has been used in genomic analysis at single-cell level, including single-cell mutation analysis in metastatic breast cancer⁸⁰, and single-cell whole exome sequencing in prostate cancer³³.

Recently, a number of microfluidic systems have been developed for affinity-capture of CTCs by introducing antibody-coated microstructures. In the earlier development, Nagrath et al. developed the first microfluidic device, CTC-Chip, that contains an array of 78,000 EpCAM-coated microposts, which are geometrically arranged in a way to promote cell adhesion under precisely controlled fluid flow (Figure 3.1A)²¹. Blood samples were processed at a rate of 1-2



Figure 3.1Antibody-coated microposts-based enrichment microfluidic devices. (A) CTC capture on EpCAMcoated microposts in CTC chip and (B) GEDI microfluidic device design. The CTC-chip consisted of an array of 78,000 microposts chemically functionalized with the anti-EpCAM antibody to capture EpCAM+ CTCs. The GEDI chip combines positive enrichment using antibody-coated microposts with hydrodynamic chromatography to minimize non-specific capture of leukocytes.

[Figure 3.1A reproduced from Nagrath et al.²¹ with permission from Nature Publishing Group; Figure 3.2B reproduced from Kirby et al.⁸¹ under the terms of the Creative Commons Attribution License]

mL/hr, and the volume that can be processed is limited to 2-3 mL. The CTC-Chip achieved recovery rate of 60 % and used to capture CTCs from patient samples with metastatic breast, colon, pancreatic, lung, and prostate cancer with CTC counts ranging from 5-1,281 CTCs/mL²¹. Another microfluidic device, known as the geometrically enhanced differential immunocapture (GEDI) chip, is developed to further increase the effective collision frequency between the CTCs and the antibody-coated microposts to increase the capture efficiency up to ~90% (Figure 3.1B)^{81,82}. The GEDI chip was used to capture CTCs from 30 castration-resistant prostate cancer (CRPC) patients on anti-prostate specific membrane antigen (PSMA)-coated microposts with median count of 54 CTCs/mL. When compared to the CellSearch[®] system, the GEDI chip detected a 2 to 400-fold increase in the number of CTCs/mL on same-day blood drawn from 25 CRPC patients⁸¹. The flexibility of GEDI chip in functionalizing microposts with different antibodies allows its broad application to different types of cancers using cancer-specific



Figure 3.2 Microfluidic devices for capturing CTCs using functionalized surfaces: (A) The herringbone chip and (B) Geometrically enhanced mixing chip. Both of these devices enhance cell-antibody collision events by inducing local mixing using structures within the microchannels.
[Figure 3.2A reproduced from Stott et al.⁷⁰ under CC BY-NC-ND; Figure 3.2B reproduced from Sheng et al.⁸⁴ by permission of The Royal Society of Chemistry]

antibodies, including anti-HER2 antibody for breast cancer⁸², and anti-MUC1 antibody for pancreatic cancer⁸³.

In order to improve the throughput of the micropost affinity methods, the surface-based microfluidic devices such as the herringbone (HB) chip⁷⁰, and geometrically enhanced mixing (GEM) chip⁸⁴ are developed to capture CTCs. Instead of microposts, these devices capture CTCs on chemically functionalized surfaces, which is suitable for larger sample processing at a faster flow rate (Figure 3.2A). The HB chip contains the herringbones structures to enhance the interaction between CTCs and antibody-coated surface for higher CTC capture efficiency and flow rate (4.5 mL/h). Using prostate-specific antigen (PSA)-coated surface, CTCs were detected



Figure 3.3 (A) The IsoFlux CTC capture system. Samples passes through an isolation zone where magnetically labeled cells are captured on the top of the channel in the presence of a magnet. After processing, the cells are retrieved inside the instrument by lifting the cap off the channel and securing it to either microfuge tube or holder to remove the target cells through pipetting (not shown). (B) The LiquidBiopsy system captures CTCs by labeling of whole blood with a functionalized ferrofluid followed by high throughput partitioning of CTC from blood using a CTC sheath flow cell. [Figure 3.3A reproduced from Harb et al.⁸⁵ under CC BY-NC-ND license; Figure 3.3B Winer-Jones et al.⁸⁶ under

the terms of the Creative Commons Attribution License]

in prostate cancer patients with localized disease (38 - 222 CTCs/mL) as well as with metastatic disease (14 to 5000 CTCs/mL)⁷⁰. The GEM chip was developed to improve herringbone mixers by increasing the groove width to reduce trapping of non-target cells, keeping a flow rate at 3.6 mL/h (Figure 3.2B). The GEM chip was applied to capture CTCs from metastatic pancreatic cancer patient samples. CTCs were found from 17 out of 18 samples (>94%) with an average count of 23 CTCs/7.5mL blood. ⁸⁴

One of limitations of the microposts and surface-based microfluidic devices is the ability of release and recover viable CTCs for downstream molecular analysis. Captured CTCs on antibody-coated microposts or surfaces typically bound tightly to the substrate, making it difficult to recover these cells for further workflow. To address this limitation, automated commercial systems that use microfluidic, immunomagnetic strategies for CTC enrichment, such as IsoFlux (Fluxion Biosciences) and LiquidBiopsy (Cyvenio) were developed^{85,86}. IsoFlux captures CTCs labeled with EpCAM coated magnetic beads on the upper surface of the isolation

cartridge under high magnetic field, while other non-labeled cells are directed to the waste channels (Figure 3.3A). The captured cells are then retrieved and can be suspended in a droplet of approximately 3 µL for direct molecular analysis. IsoFlux achieved higher capture efficiency compared to CellSearch in prostate cancer patient samples, 95 % (21/22) and 36 % (8/22), respectively⁸⁵. Similar to IsoFlux, LiquidBiopsy (Cyvenio) uses a multilayer sheath flows to capture CTCs labeled with magnetic nanoparticles (Figure 3.3B)⁸⁶. The labeled blood sample is introduced to the middle layer, and target cells are deflected and captured on the upper glass slide under the magnetic field. The LiquidBiopsy system can process relatively large volumes (5 mL/h) of whole blood, while recovering CTCs at purities greater than 1 %, enabling the direct downstream molecular analysis such as next generation sequencing⁸⁶.

Although affinity-based CTC isolation techniques described above have successfully demonstrated its ability to capture CTCs, a fundamental flaw in this approach is its reliance on the expression of EpCAM on tumor cells. While EpCAM has demonstrated to be an effective marker for capturing CTC originated from solid tumors, some tumor cells with low or no EpCAM expression could be missed when using antibodies against EpCAM. CTCs may have gone through epithelial-to-mesenchymal transition (EMT), which is associated with a loss of expression for epithelial marker, such as EpCAM^{87–90}. As a result, the most aggressive tumor cells may be the least likely to be captured using EpCAM. Furthermore, the use of specific surface antigens is less desirable because of the heterogeneity of expression of the target antigens from different, or even the same, types of tumors expressing these antigens^{91–94}. One strategy to overcome the potential bias introduced by EpCAM-based capture is to use the multiple panels of antibodies for CTC enrichment. Andreopoulou et al. demonstrated that enrichment with anti-

MUC1 combined with EpCAM can increase the sensitivity for CTC detection in metastatic breast cancer patients⁹⁵. Alternative approaches to deplete the non-tumor cells instead of positively captures CTCs expression certain antibodies will be discussed in the next section.

3.1.1.2 Negative Selection

A negative selection by depletion of unwanted cells, such as leukocytes, may offer the alternative strategies to overcome the limitation of epithelial marker-based enrichment. Using this approach, Stemcell Technologies developed the EasySep Human CD45 depletion kit, which contains magnetic nanoparticles coupled with anti-CD45 antibody targeting leukocytes⁹⁶. Labeled blood samples are incubated in the magnet cage, and unlabeled cells are poured out while target cells remained in the tube. Using the EasySep Human CD45 depletion kit, CTCs from various epithelial cancers were successfully enriched^{96,97}.

In addition to the commercialized depletion platform, a microfluidic chip, CTC-iChip, that can selectively operate in two immunomagnetic sorting modes to isolate CTCs is developed⁹⁸. The CTC-iChip can function as either positive selection mode (^{pos}CTC-iChip), where CTC are enriched by labeling them with EpCAM magnetic beads, or a negative selection mode (^{neg}CTC-iChip), where leukocytes, labeled with the common leukocyte antigen CD45 and the granulocyte marker CD15 magnetic beads, are depleted from the blood sample. Using a negative selection mode, CTC-iChip system successfully captured CTCs in patients with triple-negative breast cancer, where CTCs express only mesenchymal markers and are unlikely to be captured by a positive selection based on EpCAM expression⁹⁹.

3.1.2 **Biophysical Property-based Enrichment**

CTC separation based on biophysical property of tumor cells are often referred as 'label-free' method as tumor cells are not 'tagged' with an antibody. This approach relies on the ability to discriminate between tumor cells and other contaminant cells (e.g. leukocytes) based on physical properties, such as size, deformability and electrical charge. Here, physical characteristics including size and deformability of tumor cells are first discussed. Next, three main types of biophysical separations of CTCs are discussed: (1) a membrane microfiltration (2) hydrodynamic chromatography and (3) dielectrophoresis (DEP).

3.1.2.1 Size and Deformability of Tumor Cells

There are three main types of cells in normal blood: erythrocytes, thrombocytes, and leukocytes. Erythrocytes (also known as red blood cells) make up about 40% of the blood's volume, and are biconcave disc shaped. Red blood cells measure about 6 to 8 μ m in diameter, and 2–3 μ m thick¹⁰⁰. Red blood cells lack a nucleus, which allows them to deform through narrow passages and then restore to its original shape quickly. Thrombocytes (also known as platelets) also do not have a nucleus and are even smaller than red blood cells. These cells are spherical, ~2 μ m in diameter, which is approximately 20% of the diameter of red blood cells¹⁰¹. Leukocytes (also known as white blood cells) have a nucleus and are generally spherical in shape in circulation. Leukocytes have typical average diameters ranging from 6 to 13 μ m^{100,102,103}. These cells are physically most similar to epithelial cancer cells, and thus they are the most common type of contaminants blood cells in CTC separation based on size.

In contrast to blood cells, the mean diameters of CTCs are often assumed to be greater than that of normal blood cells, especially leukocytes. This assumption is originated from the observed diameter of cultured tumor cells (e.g. MCF-7, HeLa, LNCaP), which are between 11 and 15 µm in average^{103–105}. In clinical samples, CTCs separated from breast cancer patients were larger compared to leukocytes and had generally elongated shape when observed using DIC microscope¹⁰¹. In addition, Lighart et al. reported that patient-derived CTCs in breast, colorectal and prostate cancer are nearly three times larger than leukocytes. However, in some cases, the differences between CTCs and leukocytes are not always clear, perhaps due to the great variability in size among CTCs and leukocytes. Allard et al. observed heterogeneity in CTC size, ranging from 4-30 μ m diameters, even among CTCs from the same patients²⁴. Our recent observation based on microscopic analysis suggested that CTCs from patients with metastatic castrate-resistant prostate cancer (mCRPC) are significantly smaller in size (a median cell diameter of 7.97 µm) compared to prostate cultured cancer cell lines¹⁰⁶. Another study also supports that CTCs sizes from prostate cancer patients were between 8 and 16 µm, where the lower limit is similar to the size of leukocytes⁷⁰. All these findings suggest that there is a significant overlap in size of CTCs and leukocytes that might hinder size-based separation process. Therefore, the validation of CTC isolation technologies based on size should be carefully performed and must include clinical samples.

On the other hand, the nuclear to cytoplasmic ratio (N/C, defined as the ratio of nuclear area and cell area with the nuclear area subtracted) may allow us to infer relative deformability. The nuclear sizes of CTCs from metastatic prostate, pancreatic and breast cancer samples is found to be greater than those of leukocytes, across different patients, ranging from 1.3-3 times the size of

the average leukocyte nucleus⁹³. As a viscosity of the cell nucleus is 10-fold greater than the cytoplasm¹⁰⁷, CTCs with a higher N/C ratio are approximately 2.5-3 fold less deformable than monocytes and lymphocytes^{108,109}. Meng et al. reported that the average N/C ratio of CTCs from 36 breast cancer patients was 4.0 while that of leukocytes was 1.22¹¹⁰. Our recent observation on prostate cancer patients suggested that average N/C ratio of CTCs was 1.43 with significant variability¹⁰⁶. The combination of greater N/C ratio and greater stiffness of the nucleus relative to cytoplasm suggest that CTCs are less deformable than leukocytes. Based on these studies, the cell deformability represents an important biophysical characteristic which can be used in label-free CTC enrichment strategies.

3.1.2.2 Microfiltration

Microfiltration is a cell separation technique that relies on flowing cell sample through an array of microscale constrictions in order to capture target cells based on size alone, or based on a combination of size and deformability. Membrane microfilters consists of a semi-permeable membrane with an array of small pores that act as a sieve to isolate cells with certain size and/or



Figure 3.4 Microfiltration for CTC separation. (A) CTCs captured using ISET and (B) ScreenCell system. The ISET filters fixed blood samples through 8-µm pores in a plastic membrane. The ScreenCell system uses similar plastic filters with a hydrophilic surface and cylindrical 7.5 or 6.5-µm pores to filter fix blood samples. [reproduced from Mu et al.¹¹⁷ under CC BY 4.0]

cell deformability. Many membrane microfilters with pore sizes around 6 to 10 μ m in diameter are proven to be effective in CTC separation. Indeed, CTCs are generally assumed to be larger than leukocytes, so most of these cells are likely pass through the filter whereas CTCs are retained. Based on these size differences, Vona et al. developed the Isolation by Size of Epithelial Tumor Cells (ISET) platform to isolate CTCs from fixed blood samples using a tracketched polycarbonate membrane filter with 8- μ m-diamter, cylindrical pores (Figure 3.4A) ¹⁰⁵. Using ISET technology, CTCs in breast, prostate and lung cancer patients were successfully captured and characterized using various downstream analysis including immunophenotyping and fluorescence in situ hybridization (FISH) directly on the membrane microfilter^{111,112}. Several studies have demonstrated a higher sensitivity of ISET system when compared to CellSearch^{®112-} ¹¹⁴. Another commercialized system, ScreenCell (Figure 3.4B), uses microfilter with a hydrophilic surface and cylindrical 7.5 μ m for fixed samples (or 6.5 μ m for live samples) to enrich CTCs in colorectal, lung, and breast cancer¹¹⁵⁻¹¹⁷.

Several microfiltration systems in microfluidic devices have been developed to capture CTCs based on size and/or cell deformability. Zheng et al. developed a 2D round pore-shaped microfilter fabricated on a single 10 µm-thick parylene-C membrane by photolithography¹¹⁸. This microfilter device was further improved to include a 3D membrane microfilter with two porous parylene-C layers; top layer contains 40-µm pore, and the bottom layer contains hexagonally arranged 8- µm pores with the 10- µm gap between the top and bottom layer (Figure 3.5A) ¹¹⁹. This special design demonstrated the ability to enrich viable CTCs from metastatic colorectal cancer patient samples. Lin et al. reported a superior sensitivity of this system over CellSearch[®] in detecting CTCs from blood samples from patients with metastatic prostate,

breast, colon, or bladder cancer¹²⁰. Instead of using a vertical configuration described above, Parsortix system (ANGLE) uses a horizontal configuration of microfilters to separate CTCs based on size and deformability (Figure 3.5B). The Parsotix microdevice consists of a stepped structure that gradually decreases the channel width to <10 μ m. Smaller and softer cells pass through the gap whereas CTCs, which are larger and stiffer, are lodged in the gap. After capturing, CTCs can be released by the reverse flow for further molecular analysis. The Parsortix system was used in various studies to enrich CTCs from metastatic and non-metastatic cancer patients, finding between 20-1474 CTCs in a study of small cell lung cancer patients¹²¹. Furthermore, the Parsortix captured significantly more CTCs than CellSearch[®] (p = 0.04) in seven prostate cancer patient samples (an average of 32.1 and 10.1 respectively)¹²².



Figure 3.5 Microfluidic microfiltration. (A) 3D membrane microfilter device and (B) Parsortix System. The 3D membrane microfilter device conscists of two porous parylene-C layers. The bottom layer contains hexagonally-arranged 8-μm pores. The top layer contains larger 40-μm pores that align with the corresponding hexagon patterns on the bottom membrane. The Parsortix microdevice has a stair-like structure that gradually decreases the channel width to <10 μm.</p>

[Figure 3.5A reproduced from Zheng et al.¹⁷⁹ under CC-NS-4.0; Figure 3.5B reproduced from Chudziak et al.¹²¹ with permission from Analyst] In general, microfiltration methods are easy to use and achieve high isolation efficiency and throughput. However, the key challenge in this system is the clogging of pores on the membrane as large numbers of cells are processed, reducing the selectivity of the separation process. Moreover, prolonged contact between the cells and the filter surface increases the potential for nonspecific adsorption; in turn, the retrieval of captured cells for further characterization is often difficult. Furthermore, the overlap in size between CTCs and leukocytes makes it difficult to achieve the high purity samples for further molecular analysis. To resolve these drawback, our group previously developed a microfluidic cell separation device based on size and deformability^{123,124}. Using oscillatory flow, cells that accumulate in the filter microstructure can be removed, diminishing clogging and reducing nonspecific adsorption.

3.1.2.3 Hydrodynamic Sorting

Hydrodynamic sorting relies on the manipulation of fluids in microscale to passively separate cells with different size. One promising type of a hydrodynamic sorting is deterministic lateral



Figure 3.6 CTC-iChip: (A) Device design and (B) integrated microfluidic system. The microfluidic CTCiChip system first sorts the various cells in a blood sample by size, allowing only CTCs and white blood cells to enter the inertial focusing chamber, which lines up those cells into a single file. A magnetic field then deflects cells previously labeled with tiny magnetic beads, isolating CTCs for further study. [reproduced from Ozkumur et al.²² with permission from AAAS]



Figure 3.7 Vortex Chip. (A, B) Design and (C, D, E) cell separation using Vortex Chip. The Vortex chip used for cell trapping consists of 8 channels in parallel with 8 reservoirs. At the channel inlet, cells are randomly distributed and experience two opposing lift forces, the wall effect and the shear-gradient lift force. As a result, particles migrate to dynamic lateral equilibrium positions, depending on the channel cross section. Upon entrance in to the reservoir, the wall effect is reduced. Larger cells (violet dashed line) still experience a large lift force and are pushed away from the channel center through the separatrix and into the vortices, where they are stably trapped. Smaller cells (red dashed line) do not experience enough lift force to cross the separatrix and remain in the main flow.

[reproduced from Sollier et al.¹³⁰ with permission from Lab on a Chip]

displacement (DLD), which uses a displacement of cells in an array of microposts under precisely controlled flow. Similar to microfiltration, the gap distance and obstacle size play an important role in separation process. In the separation of cells using DLD, cells below a critical size will follow streamlines and pass through an array of posts with no net lateral displacement. When cells are bigger than the critical size, they will collide with the obstacle, shifting to a different streamline from the original streamline¹²⁵. Using this technique, Davis et al. demonstrated that whole blood could be continuously separated into red blood cells and white blood cells in an array of circular posts with 10 µm gap¹²⁶. This design is further optimized by replacing circular posts with triangular ones with gap size 42 µm in order to accommodate lager tumor cells for higher isolation efficiency¹²⁷. The device was validated using the spiked breast cancer cells (MDA-MB-231) in whole blood, demonstrating high isolation efficiency (<85%) at a flow rate of 10mL/min with no effect on cell viability¹²⁷. Another microfluidic device, CTCiChip, uses the combination of DLD and antigen dependent sorting of CTCs (Figure 3.6)⁹⁹. The CTC-iChip composed of two separate microfluidic devices: In the first device, DLD is used to remove red blood cells from whole blood with an array of droplet-shaped posts with 32-µm gap. In the second device, magnetic beads labeled with either tumor-specific or leukocytes-specific antibodies are used to target either positively or negatively enrich CTCs. In application of patient samples from various cancer types, CTCs were successfully captured in a positive selection mode with a mean CTC count 3.2 CTCs/mL, demonstrating a higher sensitivity compared to the CellSearch[®] system (a mean CTC count 1.7 CTCs/mL).

Besides DLD, inertia-based microfluidic platforms to isolate CTCs have also been reported. Inertial focusing passively separates CTCs from other blood cells based on size by exploiting the effects of two inertial lift forces: (i) shear-gradient lift force that directs particles towards the channel wall, and (ii) a wall effect lift force that directs particles away from the channel wall. In rectangular channels, the combination of these inertial forces leads to the migration of particles to their equilibrium positions based on their sizes¹²⁸. Using the inertial focusing and "expansion-contraction" trapping reservoirs along microchannels, Hur et al. developed a continuous microfluidic platform to trap larger cells (e.g. CTCs) while smaller cells flush along the



Figure 3.8 ClearCell system. (A) Illustration of the design of a multiplexed device, (B, C) optical image of an actual multiplexed spiral microfluidic device for capturing CTCs with two inlets and two outlets. Blood sample and sheath fluid are pumped through the device using two separate syringe pumps. Under the influence of inertial lift and Dean drag forces in the fluid flow, CTCs focus near microchannel inner wall (Region A-A) while WBCs and Platelets goes through one Dean cycle and migrate back towards the outer wall (Region B-B), thus achieving separation.

[reproduced from Khoo et al.¹³³ under CC0]

microchannel to the outlet (Figure 3.7)¹²⁹. A commercialized version of this device, the Vortex Chip, consists of eight channels in parallel with 8 reservoirs for CTC trapping, and can process the whole blood samples at a rate of 7.5 mL/20 min¹³⁰. Using Vortex Chip, CTCs were successfully captured in all 12 samples from breast (n=4) and lung (n=8) cancer with a relatively high purity $(57-94\%)^{130}$.

In a spiral microchannel, the curvature design generates rotational flow within the channel, and thus creates two symmetrical counter-rotating vortices across the channel cross section, referred as Dean vortices¹³¹. A combination of inertial forces, coupled with Dean vortices result in particles to position at their preferential equilibrium points in a spiral microchannel. The larger cells (e.g. tumor cells) will move towards the inner wall while the smaller cells (e.g. white blood cells and red blood cells) will migrate to the outer half of the channel, resulting in the formation of distinct streamlines based on size¹³². Using this approach, a spiral microfluidic chip with

rectangular cross section has been developed to isolate CTCs from metastatic lung cancer patients, ranging from 5 to 88 CTCs/mL at a rate of 3 mL/h¹³². Commercial version of this device, ClearCell FX (Cambridge Biomedics) detected CTCs in patients with metastatic breast and lung cancer, ranging from 12-1275 CTCs/mL and 10-1535 CTCs/mL, respectively, with an improved throughput at 7.5 mL/10min (Figure 3.8)¹³³.

In general, the hydrodynamic sorting system allows the high throughput and capture efficiency without clogging issue. Additionally, the inertial focusing exerts minimal stress on cells, enabling the capture of viable CTCs for sensitive downstream analysis. However, the hydrodynamic sorting is mainly based on cell size, and thus it may not be effective in sorting some cancer cell types, in which the size differences between CTCs and leukocytes are not clear.

3.1.2.4 Dielectrophoresis (DEP)

Apart from membrane microfilters and hydrodynamic separation, dielectrophoresis (DEP) offers the contactless cell manipulation based on dielectric properties. Dielectrophoresis (DEP) refers to the movement of cells in the non-uniform electric field¹³⁴. When cells are subjected to non-uniform electric field, a dipole moment on the cell is induced due to the electrical polarization at the cell's membrane with the surrounding solution. This dipole moment creates a translational movement of cells to achieve electrostatic equilibrium¹³⁴. The polarizability of cells depends strongly on their composition, morphology, phenotype and the applied electric field frequency; therefore, cells of different types or physiological states can potentially be discriminated by the DEP.

Depending on the conductivity and permeability of the cell as well as surrounding medium, the cells exhibit either attractive or repulsive response at a given electric field frequency, causing them to move towards (termed as positive-DEP, pDEP) or away (termed as negative-DEP, nDEP) from the electrical field source¹³⁵. Using this strategy, Wang et al. introduced an efficient cell separation system combined with field-flow-fractionation (FFF)¹³⁶. In this design, cells with distinct electric properties are positioned at different heights due to DEP forces. Subsequently, cells at different heights were carried by field flow at different velocities, and thus separated. This DEP-FFF device demonstrated an efficient separation of breast tumor cells from normal lymphocytes. Furthermore, a commercial DEP based microfluidic platform, ApoStream (ApoCell), was launched to separate CTCs in clinical samples (Figure 3.9)^{137,138}. In this design, CTCs are attracted to the electrodes (pDEP) on the bottom of the flow channel whereas leukocytes are repelled (nDEP) towards the center of the channel when cells are subjected to electric field. Using ApoStream system, CTCs from patients with breast, ovarian, and lung cancers were isolated with an average CTC counts of 9, 2, and 139 respectively¹³⁹. When compared to the CellSearch[®] system, the ApoStream captured higher CTC counts (0-8 CTCs/7.5mL for CellSearch[®] and 3-487 CTCs/7.5mL for ApoStream), demonstrating a higher sensitivity.

Recently, a new technology DEPArray (Silicon Biosystems) is developed for single-cell recovery based on DEP strategy^{140,141}. The DEPArray contains an array of more than 300,000 electrodes that trap a single cell using nDEP forces, which can keep cells in levitation. In this way, thousands of cells can be suspended in stable levitation and manipulated independently. In fact, each electrode is programmable, allowing the selective movement of each individual cell along any path in the whole array. DEPArray is often combined with pre-enrichment method such as the CellSearch[®] system for isolating single CTCs. For instance, DEPArray was used to



Figure 3.9 Schematic representations of ApoStream device. (A) ApoStream prototype instrument, (B) 3D CAD model of the flow chamber, and (C) DEP enrichment for CTCs. The sample injection port introduces cells at the bottom of the flow chamber upstream of the buffer. Electrodes line the bottom of the flow chamber and generate an electric filed that attracts CTCs to the chamber flow and repels leukocytes towards the center of the channel.

[reproduced from Balasubramanian et al.¹³⁸ under CC0]

isolate single CTCs in patient samples with metastatic breast cancer initially enriched by CellSearch^{®142}. In this study, overall 221 single CTCs were sorted from eight patients and 115 were successfully sequenced for mutational analysis, demonstrating a potential for sorting pure CTCs extracted by a combination of CellSearch[®] and DEPArray.

3.1.3 Summary

In summary, there are two general classes of CTC separation technologies; affinity-based and biophysical property based methods. Affinity-based methods rely on the expression of surface makers on target cells whereas biophysical property based methods separation tumor cells based on the distinct tumor cell characteristics such as size, deformability and electric charge. The affinity based methods provides a highly specific separation of CTCs with less leukocyte contamination. However, this technique has its limitations on using specific antigen expression to capture CTCs because CTC consists of a very heterogeneous population. Label-free methods can overcome this limitation by isolating CTCs based on their physical properties in an unbiased manner independent of surface marker expression. In addition, many hybrid systems have been developed including a combination of affinity-based capture with other principles such as size-based capture or DEP system, demonstrating a potential to overcome the limitations associated with both these systems individually.

3.2 Single Cell Isolation Techniques

Genome sequencing of individual CTCs is particularly challenging because of the extreme rarity of CTCs, even after affinity-based or biophysical-based enrichment, which invariably produce an impure sample with contaminate leukocytes. Here, we review available techniques for single cell isolation and genome sequencing of single CTCs analysis are discussed including fluorescence activated cell sorting (FACS), micromanipulation, and laser capture microdissection (LCM). The principle of each method as well as the advantage and limitation of their applications are discussed.

3.2.1 Fluorescence Activated Cell Sorting (FACS)

Fluorescence activated cell sorting (FACS) identifies and separates cells labeled with fluorophore-conjugated antibodies that recognize specific surface markers on target cells¹⁴³. As the suspended labelled cells are passed through the cytometer, the fluorescence detector identifies cells based on the cell specific signals. Based on their characteristics, the droplet containing a cell of interest is sorted into appropriate collection tubes for later analysis. FACS has been widely used for isolation of highly purified cell population as this method is a very robust cell sorting technology with high-throughput (50,000 cells/sec)^{144,145}. However, it requires cell sample consists of $\sim 1 \times 10^5$ cell population and containing more than 0.1% positive cells which limits its application to isolation of rare cells including CTCs¹⁴⁶. Thus, it is not surprising that only limited studies have been reported to isolate single CTCs using FACS. In one study, single CTCs from lung cancer patient samples were isolated using FACS (FACSARIA II, BD Biosciences)¹⁴⁷. Another study reported the isolation of CTCs from breast cancer patient samples using FACS (MoFlo XDP cell sorter)¹⁴⁸. In both studies, the samples were pre-enriched using the CellSearch[®] system, implying the limitation of FACS method to apply in direct single CTC isolation.

3.2.2 Micromanipulation

Micromanipulation is a manual cell picking method that aspirates single cells using negative pressure while under observation using an inverted microscope¹⁴³. Cell samples in a dish or well-plate are visually inspected for identifying the target cells. The aspirated liquid, including the cell of interest, can be transferred to the collection tube or a well-plate for further analysis. This process is typically operated manually, which is laborious and time-consuming. Despite its drawbacks, micromanipulation system has been most commonly used to isolate single CTCs after initial enrichment. Ni et al. reported single lung cancer CTC isolation from pre-enriched samples by the CellSearch[®] system for copy number variation study³¹. Another study performed whole-exome sequencing of single CTCs from metastatic prostate cancer patient samples that were pre-enriched by MagSweeper³³. Nonetheless, the number of single cTCs for prostate cancer due to the inherent nature of slow and laborious manual picking process^{31,33}.

3.2.3 Laser Capture Microdissection

Besides FACS and micromanipulation system, the laser capture microdissection (LCM) has been widely used to isolate single cells from mostly solid tissue samples, which are typically provided fixed in formalin, embedded in paraffin, or cryo-fixed¹⁴³. Similar to micromanipulation method, a tissue section is observed through an inverted microscope to identify target cells. The target cells are marked by drawing the line around it, followed by laser cutting and extraction. While the cutting procedure using laser is mostly the same, there are two main types to extract target cells using either infrared (IR) or ultraviolet (UV) laser (Figure 3.10).

The Arcturus LCM system performs 'contact-based' extraction of single cells using IR laser. In this system, the samples are typically stained for identification of target cells^{143,146,149}. Once target cells are identified, the IR laser activates a transfer film on a cap, located directly above the target region. The cells of interest are then adhering to the film on a cap, and then transferred to a microcentrifuge tube for further application. On the other hand, the PALM Zeiss system is a 'contact-free' method, isolating single cells using UV laser beam^{143,146,149}. The samples are prepared on a glass slide, layered with a thin and transparent membrane. When target cells are



Figure 3.10 Laser capture microdissection systems. [reproduced from Vandewoestyne et al.¹⁴⁹ under CC BY-NC-ND 3.0]

identified, UV laser beam cuts around the region of interest. The desired cells are then catapulted against gravity into a cap of the collection tube using a short defocused UV laser pulse. The thin membrane acts as the acts as a stabilizing backbone during lifting. The collected samples can be visually confirmed on a collection tube by moving the sample stage to focus the samples collected on the cap.

While LCM system has been extensively used in tissue samples, some studies have been reported to extract CTCs using LCM combined with membrane microfiltration methods. In membrane microfiltration methods, CTCs are captured based on size and fixed on a membrane for identification, which serves as a tissue substrate. For example, CTCs were isolated from liver and breast cancer patient samples using Arcturus LCM system for further characterization such as PCR and sequencing^{150,151}. In these studies, CTCs were captured on a microfilter membrane with 8-µm pores (ISET system), served as the tissue substrate. Captured cells were isolated using Arcturus LCM system, obtaining the high purity sample for mutation analysis.

Recently, a microfluidic device that captures CTC on polymer nanofiber (PN)-NanoVelcro substrate was developed to isolate single cells using LCM system^{152,153}. First, the PN-NanoVelcro substrate is prepared by electrospinning method on a membrane glass slide. A microfluidic channel that are functionalized with anti-EpCAM antibodies are placed on top of the PN-NanoVelcro substrate to capture CTCs. Afterward, the CapSure HS Cap (Acrctux system) is placed directly above the region of target cells. Then, IR laser beam was applied to melt the polymer membrane on the cap, resulting the conical polymer pillar, also called sticky finger, adhering onto the PN-NanoVelcro substrate. In the following, the UV laser is activated to

cut the substrate to collect single CTC for subsequent molecular analysis. This system was used to isolate 99 single CTCs from 5 blood samples in prostate cancer⁷³. These single cells were further analyzed using whole genome sequencing along with tissue sections, finding that CTCs and tumor tissues shared somatic single nucleotide variations and rearrangements.

3.3 DNA Sequencing of Single CTCs

Single-cell analysis of CTCs is a challenge because each cell has only 6 pg of genomic DNA, requiring high sensitive and efficient amplification step for genomic profiling such as next generation sequencing (NGS)¹⁴⁶. Several whole genome amplification (WGA) techniques have been developed to overcome the low input issue, enabling the generation of sequencing libraries



Figure 3.11 NanoVelcro chip. (A) The configuration of PLGA nanofiber (PN)-NanoVelcro Chip, (B) a SEM image of the electrospun PLGA nanofibers, and (C) schematic illustration of the workflow for isolation of CTCs.

[reproduced from Zhao et al.¹⁵³ with permission from Advanced Materials]

from single cells with >90% coverage^{154–157}. Combined with the advances in WGA and single cell isolation technologies, several single cell analysis of CTCs have been reported in various cancer types including $lung^{31}$, colorectal³⁰, breast¹⁵⁸ and prostate cancer.

In a recent study, single CTCs from lung cancer patients were sequenced to examine copy number and single-nucleotide variation³¹. CTCs from 11 lung cancer patients were pre-enriched using the CellSearch[®] assay, followed by single cell isolation using a micromanipulator (TransferMan NK2). Total 37 single CTCs were sequenced using the targeted next generation sequencing (Illumina HiSeq/MiSeq). Ni et al. reported that several tumor-related genes, including the mutations associated with drug resistance (e.g. EGFR, PIK3CA) were frequently mutated not only in single CTCs but also in the primary and metastatic tumor. They also found that single nucleotide variation detected in single CTCs are highly heterogeneous. Moreover, they reported the stable reproducibility in the copy number variation(CNV) patterns of CTCs at different time point during drug treatment, suggesting that CTCs can be used to determine the disease progression over time without invasive repeated biopsies.

Similar study was performed in colorectal cancer to investigate strategies for copy number analysis and NGS of single CTCs³⁰. In this study, CTCs were pre-enriched in 21 of 37 patients (range, 1-202/7.5mL blood) using the CellSearch[®] system. Overall 37 intact CTCs from six patients were isolated using a micromanipulator (TransferMan NK2), and analyzed using arraycomparative genomic hybridization (aCGH) and NGS (Illumina MiSeq). First, using aCGH, multiple colorectal cancer-related copy number variations were found in single CTCs, many of which were also present in the respective primary tumor. Subsequent deep sequencing, targeting

68 colorectal cancer- related genes, revealed the driver genetic mutations (e.g. APC, KRS, and PIK3CA) in single CTCs. Interestingly, most of these mutations were also present in primary and metastatic tumor tissue samples, implying the value of CTCs in clinical application for non-invasive disease monitoring.

Another mutational analysis was performed on single CTCs from metastatic breast patient samples using NGS in order to investigate cell heterogeneity and provide a tool for a personalized medicine approach¹⁵⁸. First, CTCs were enriched and enumerated by CellSearch[®] from four metastatic breast cancer patients, and individually isolated by DEPArray. Overall 14 single CTCs was sequenced using NGS (Ion Torrent PGM system), targeting 207 amplicons covering mutations from 50 oncogenes and tumor suppressor genes. In total 51 sequence variants in 25 genes of the panel were found including PIK3CA, PTEN, and TP53. The highest number of somatic deleterious mutations (8 mutations) was found in gene TP53, which mutation is associated with more aggressive disease and worse overall survival in breast cancer. The major part of somatic mutations was usually detected in only 1 single CTC from 1 patient, demonstrating a high intra- and inter-patient heterogeneity. Furthermore, mutational profiles of CTCs before and after treatment shared only few sequence variants, suggesting that CTC characterization may be applied to monitor the response to therapy.

In the prostate cancer study, Lohr et al. established a standardized process for whole exome sequencing of single CTCs³³. CTCs were first enriched by immunomagnetic method with anti-EpCAM magnetic beads (MagSweeper), and then single CTCs were isolated by automatic micromanipulation for whole genome amplification. In this study, 25 single CTCs and multiple

spatial tissues of the primary prostate tumor and bone metastases from two prostate cancer patients were sequenced using whole exome sequencing method. More than half of the mutations detected in the primary and tumors were matched in the CTC populations. Furthermore, a larger number of CTC-specific mutations were found, demonstrating the important values of CTCs in metastatic disease. Another study of single CTCs from a castration-resistant prostate cancer (CRPC) patient under chemotherapy was performed to predict treatment efficacy in real time over the course of cancer treatment³². In this study, CTCs were enumerated by the enrichmentfree CTC detection platform (High Definition-CTC, Epic Sciences) based on the multi parameter fluorescence imaging. CTCs were stained for cytokeratin (CK) for identification, coupled with evaluation of androgen receptor (AR) status. Following the enumeration, 41 single CTCs were isolated using micromanipulation (TransferMan NK2) and sequenced using whole genome sequencing technique. Copy number variation in respond to therapy were detected at the genomic level, indicating that sequential characterization of CTCs can be useful in predicting treatment efficacy and monitoring disease evolution in individual patients.

In summary, several studies have been reported for single CTC analysis in various cancer types such as lung, colorectal, breast and prostate cancer. Different approaches were used to enrich and isolate single CTCs in these studies, including a combination of affinity-based enrichment (e.g. CellSearch[®]) with micromanipulation for single cell isolation. Single-cell isolation step still presents a 'bottle-neck' of the whole workflow as the most common method (e.g. micromanipulation) is very laborious and time-consuming. Nevertheless, available sequencing technologies with whole genome amplification are now sensitive enough to allow us to study CTCs at single-cell level. Several studies presented that single CTC sequencing can provide a
useful information on disease status and drug efficacy. Therefore, single-cell analysis of CTCs seems to hold promise for future clinical applications by focusing on non-invasive disease management and aiming for personalized therapy.

Chapter 4: Morphology Study of CTCs in Prostate Cancer

4.1 Introduction

Emerging label-free CTC capture strategies distinguish CTCs based on their assumed biochemical characteristics. It is broadly accepted that CTCs have distinct biophysical properties, including larger size than leukocytes, greater nuclear to cytoplaymic ratio, as well as distinct nuclear and cell morphology¹⁵⁹. In fact, this biophysical difference has become the premise underlying development emerging 'label-free' enrichment systems including microfluidic filtration, hydrodynamic cell sorting, and dielectrophoresis, which report highly efficient tumor cell capture^{118,132,134}. While these systems capture tumor cells at high efficiency, they are typically validated using cultured tumor cells rather than CTCs, because of the extreme rarity and low availability of CTCs. It is possible that the performance of these enrichment systems is misrepresented because, while CTCs may resemble cultured tumor cells, they may exhibit distinct morphological characteristics, depending on the originating tumor¹⁶⁰. In fact, there is little evidence available comparing the morphological properties of CTCs with cancer cell lines and this prompted us to perform a systematic comparison of the morphological properties of CTCs, isolated from patients with castration-resistant prostate cancer (CRPC) using the CellSearch[®] system, to standard prostate cancer cell lines.

The rest of the chapter will be organized as follows: Section 4.2 describes the general approach used in this study. Section 4.3 presents the detailed experimental process including CTC isolation using CellSearch[®], imaging processing, as well as size and nuclear to cytoplasmic ratio measurement. Section 4.4 presents the results from the morphological studies on CTCs and

cultured cancer cells, including cell size, eccentricity and nuclear cytoplasmic ratio. The chapter concludes with a summary in Section 4.5.

4.2 Approach

The purpose of this study is to analyze the morphological differences between CTCs from patients with prostate cancer and cultured human prostate cancer cells, using images from the CellSearch[®] system. This analysis will assess whether cultured cancer cells represent a suitable model for CTCs when validating CTC separation and characterization methods. In order to investigate the morphological properties of cancer cells, we developed a software to examine the morphologies of both patient CTCs and model prostate cancer cell lines, following CellSearch[®] enrichment. Specifically, we compared the size, eccentricity and nuclear cytoplasmic ratio between CTCs from 16 metastatic castration-resistant prostate cancer (CRPC) patients and four human prostate cancer cell lines including LNCaP, DU145, C4-2 and PC3.

4.3 Experimental Section

4.3.1 Sample Preparation and Cell Culture

Blood samples from healthy donors and patients with metastatic castrate resistant prostate cancer (CRPC) were collected in 10 ml CellSave Vacutainer tubes (Veridex) containing proprietary anticoagulant and preservatives. Patient specimens were collected from donors diagnosed with CRPC, who ranged from 53–83 years, and had serum prostate specific antigen (PSA) levels, ranging 21.1-2200 μ g/L (Table A1).

Human prostate cancer cell lines including LNCaP (ATCC: CRL-1740), DU145 (ATCC: HTB-81), and C4-2 (ATCC: CRL-1595) were maintained in culture using RPMI-1640 medium (HyClone, Logan, UT) with 10% fetal bovine serum at 37°C with 5% CO2. PC3 (ATCC: CRL-1435) cells were cultured similarly, but using DMEM (HyClone, Logan, UT) medium. Cultured cancer cells were doped into 7.5 ml of blood from a healthy donor into CellSave[®] Vacutainer tubes and processed within 48 hours identically as the patient samples.

4.3.2 Isolation and Enumeration of CTCs by CellSearch Assay

CTC isolation and enumeration were performed using the CellSearch[®] system as previously described⁷. Briefly, blood samples were drawn into 10-ml CellSave Vacutainer tubes (Veridex) containing proprietary anticoagulant and preservative. Samples were maintained at room temperature and processed within 48 hours of collection. The CellSearch[®] system captures EpCAM expressing cells using antibody-coated magnetic beads and then labels these cells with fluorescent dyes, such as DAPI, CD45, and cytokeratins (CK), in order to distinguish potential CTCs from leukocytes. After immunomagnetic capture and fluorescence staining, images of candidate CTCs are obtained in brightfield and three fluorescence channels (DAPI, CD45, and CK). The captured images are segmented into multiple smaller images each containing a single cell and reassembled in a panel in software. Finally, a certified technician positively identifies the CTCs by reviewing the size, shape, and fluorescence intensity of each candidate cell.

4.3.3 Imaging Processing for Cell Size and Shape Measurement

To study the morphology of CTCs and cultured cancer cells, we exported images of individual cells from the CellSearch[®] system and analyzed them using a software program we developed

using LabView (National Instruments, Austin, TX) (Figure 4.1). The images were square matrices with sizes ranging from 80 to 200 pixels and formatted as portable network graphics (PNG) files as 8 bit mono or 24 bit color composites.

To calculate area in pixels, the images were initially processed using cluster thresholding to detect bright objects to match the auto-exposure performed by the CellSearch[®] system as shown in Figure 4.2A. Particles with pixels in contact with the edge of the image frame were excluded



Figure 4.1 A screen shot of image analysis software developed by Richard R. Ang using LabView. The program acquires the images for each CTC candidate. A selected image (highlighted in yellow) is analyzed to measure the area in pixels. An ellipse is fitted to this image and overlaid on top of the original image for checking. Parameters for intermediate image processing steps, as well as statistics for the whole collection are also displayed.



Figure 4.2 Data processing algorithm for the image analysis software developed by Richard R. Ang. Images acquired from the CellSearch® are analyzed via to determine (A) the overall area, and (B) the best-fit ellipse.

to eliminate cells incompletely bounded by the images. Debris particles were removed using two-iterations of 3x3 erosion particle filter. Cell and nuclear size was determined by counting the number of above-threshold pixels in the cytokeratin and DAPI channel, respectively. Results for the cell and nuclear size calculation were filtered to remove cells with improbable nuclear sizes, wherein the nuclear area exceeding 95% of the cell area. This filtration steps rejected 209 out of



Figure 4.3 Rejected cells from CTC identification. These images of cell fragments commonly appeared during image analysis and were not included in the CTC count or cell size measurements. Typical CTC fragments include a nucleus partly covered by cytokeratin, or a nucleus completely separated from cytokeratin. These fragments likely originated from CTCs undergoing apoptosis. The yellow scale bar is 5 μm in length.

732 images, or 28.5% of the total. The majority of the rejected images (Figure 4.3) contained cell fragments or poor quality images.

To measure the eccentricity of cell shape, an ellipse was fitted to the outline of the cell (Figure 4.2B). To enhance detection of the cell outline, the images were contrast-enhanced before autothresholding. Two iterations of 3x3 erosion particle filter, as well as single dilate filter were performed the edges of the particles. An ellipse was fitted using the contour tracing method to search for the longest ellipse perimeter within the image. After fitting the ellipse, the results were visually confirmed by the operator. The elongation factor (EF), defined as the ratio between the major and minor axes of the best-fit ellipse, was calculated to quantify eccentricity of the cell shape.

4.3.4 Size Measurement Calibration

To calibrate size measurements from the CellSearch[®] images, we separately measured the size of the cultured prostate cancer cells in suspension using the CEDEX XS image-based cell analyzer (Roche, Germany). Grown cultured cancer cells are trypsinized and resuspended in the culture medium. Cell counts were evaluated using a 1:1 dilution of cell suspension in trypan blue (Gibco, Grand Island, NY). A 10 μ l of cell suspension is loaded on the Smart Slide (Roche, Germany), and then read to measure the cell diameter. The conversion factor from pixels to micrometers can be determined using the following equation,

$$Conversion Factor = \frac{A_{CEDEX}}{A_{CellSearch}}$$
(4.1)

The size of CTCs from patient samples was estimated by products of the conversion factor and area of CTCs measured from CellSearch[®] images.

4.3.5 Nuclear Cytoplasmic Ratio Measurement

After calibration, the nuclear cytoplasmic ratio is calculated as the ratio of nuclear area to cytoplasmic area as below. The nuclear cytoplasmic ratio (N/C) ratio is defined as the ratio of nuclear area (A_N) to cytoplasmic area (A_C), where A_C is considered as the area of the cell excluding A_N .

$$N/C Ratio = \frac{A_N}{A_C}$$
(4.2)

4.3.6 Sample Selection

Patients with CRPC were recruited by BC cancer agency, and gave written informed consent before the study began. In all, we included 83 individuals who were chemotherapy-naïve and enrolled onto a randomized phase II clinical trial of a novel agent¹⁶¹. We collected all of the images showing DAPI+CK+CD45- events, generated by CellSearch[®] analysis. Based on the likelihood that a small fraction of these events represented legitimate CTCs, we restricted our analysis to only those patients with >40 DAPI+CK+CD45- events. This criterion reduced the number of patients in the analysis to 19, and a further three patients were excluded due to low quality of CellSearch-acquired images. CTC enumeration was independently performed for the remaining 16 patients by a CellSearch-qualified technician and the CTC counts ranged from 11 to 106 CTCs/7.5m, with a median value of 41.5 CTCs/7.5ml. After excluding unsuitable images by the filtration steps using our software (Figure 4.3), a total of 523 CTCs from mCPRC was analyzed along with 800 cultured cancer cells from the four prostate cancer cell lines.

4.4 Results and Discussion

4.4.1 Cell Size

Analyzing images of cell processed using the CellSearch[®] system and calibrated against standard microscopy, we found significant size differences between CTCs from patients with prostate cancer and cultured prostate cancer cells (Figure 4.4). Specifically, the average diameter of prostate cancer CTCs among our 16 patients, is just over half that of the cultured cancer cells, with 7.97 ± 1.81 µm for CTCs and 13.38 ± 2.54 µm for cultured cancer cells (p<0.001), respectively. This finding is consistent with a study by Coumans and colleagues¹⁰³, who reported that prostate CTCs were smaller than those of breast or colorectal cancers. This study also estimated that prostate cancer CTCs were ~25% larger than our current report. This discrepancy likely resulted from their use of a Coulter pipette for size calibration, which was less precise than image analysis for small length scale (<10 µm) size estimates. Furthermore, our estimate of cell



Figure 4.4 Diameters of CTCs from prostate cancer patients (pre-treatment) and cultured prostate cancer cells. The average diameter of CTCs (7.97 μ m) was significantly smaller than cultured cancer cells (13.38 μ m) (p<0.001).

diameter is consistent with the small mean cell volume reported by Ligthart and colleagues¹⁶², as well as another recent study showing LNCaP total cell area is 1.6-fold greater than that of prostate cancer $CTCs^{160}$. It is also interesting to note that the optimal pore size used in previous studies for filtration based capture of CTCs was 8 μ m, which coincides with our estimated cell diameter of 7.97 μ m^{103,115,118,120}. Micropore filtration techniques have reported as high as 90% CTC recovery, but relatively low sample purity. The similarity in size between CRPC CTCs investigated in this study and leukocytes may represent a key limitation of filtration-based methods. This limitation can be potentially overcome by enrichment methods that combine size and deformability of cells^{163,164}.

We also considered the possibility of our patient selection criteria (CTC count >40) may have biased for a greater number of smaller CTCs. While patients in this study were chemotherapynaive, they would have participated in a range of therapeutic interventions and would represent patients in the late stage of the disease. Due to these or other unique physiological burdens



Figure 4.5 Correlation between cell size and CTCs count. There appeared to be no correlation between CTC cell size and cell count for CTCs identified by CellSearch from patients with metastatic castrate resistant prostate cancer. The cell size ranged from 6.9 μm to 8.95 μm; while the CTC count varied from 11 to 106.

within our patient cohort, caution should be exercised in generalizing these results to all CRPC CTCs. However, we observed no correlation between CTC cell size and cell count (Table A1 and Figure 4.5). Interestingly, while other studies have reported a high degree of heterogeneity in CTC cell size^{118,162,165,166}, our size estimation based on microscopic analysis demonstrated that the inter-patient variation of the mean cell size was quite small, ranging from 7.05 μ m to 8.94 μ m with a median of 8.04 μ m. Furthermore, the currently accepted criterion used by the CellSearch[®] system to validate CTCs is that their size must be larger than neighboring leukocytes¹⁵⁹. However, this size definition was largely determined based on CTCs derived from breast cancer^{110,167–169} that have a median cell diameter of 13.1 μ m¹⁰³. Our observation that CTCs from patients with CRPC are significantly smaller in size (~8 μ m) suggests that these conventional criteria for CTC identification may underestimate the true CTC count.

Our finding that prostate cancer CTCs are consistently smaller than cultured prostate cancer cells is potentially important for emerging label-free CTC enrichment strategies. First, size-based isolation of CTCs may have limited efficacy for the capture of the smaller CTCs found in CRPC patients because they will not be as clearly separated from leukocytes. Typically, larger cancer cells, such as HELA (>20 μ m), LNCaP (18 μ m), MCF-7 (>15 μ m), MDA-231 (15 μ m)^{103,170,171}, are used to demonstrate the efficacy of these techniques. Other cultured cancer cells, such as L1210 mouse lymphoma cells (10 μ m), with smaller diameter may represent better models for CTC enrichment¹⁷². Second, the contribution of the nucleoplasm to cell stiffness is 10-fold greater than the cytoplasm¹⁰⁷; CTC enrichment techniques that capture CTCs based on size and deformability may prove to be superior to those that sort based on size alone.

4.4.2 Cell Shape

Cancer cell lines are typically used in spiked-in experiments to validate various CTC isolation techniques. The common pre-treatments of cultured cancer cells with trypsin, to dissociate them from tissue culture flasks, or sample processing using the CellSearch[®] assay may also dramatically influence the cell shape. Through comparison of trypsin-treated cultured cells and CRPC CTCs, following CTC enrichment, we evaluated whether these cultured cells are appropriate models for patient CTCs. As shown in Figure 4.6, CTCs exhibited significant shape variability with many cells having a more elongated shaped, while cultured cells were generally round in shape. We quantified the eccentricity of the cell shape using the elongation factor (EF), defined as the ratio between the major and minor axes of the best-fit ellipse. As shown in Figure



Figure 4.6 Example images of cultured prostate cancer cell (A–D) and CTCs from prostate cancer patients (E–L) captured using the CellSearch[®] system. CTCs were noticeably smaller than cultured cancer cells (A–

D). Cultured cancer cells were mostly round with regular cell and nuclear shapes. The nucleus was typically centered and surrounded by cytokeratin (E–L). CTCs exhibited highly variable shapes, including round (E), oval (F), elongated (G–J), and clusters (L). Non-round and multi-nucleate cells were sometimes observed (G–K). The yellow scale bar is 5 µm in length.

4.7A, CTCs were significantly more elongated than culture cancer cells with an average EF of 1.27 compared 1.17 for cultured cancer cells (p<0.05). Our observation is consistent with previous reports on substantial variability in the size, shape of cells and/or their nuclei^{103,115,162,166}. One possible reason for diversity in cell morphology are apoptotic events associated with CTC dissemination¹⁷³. Other previous reports also suggested that the cytomorphological changes observed in CTCs may represent functional changes associated with vascular transport^{174,175}. It is interesting to note that cytomorphological abnormality of CTCs has been correlated to poor clinical outcome in metastatic breast, colorectal, and prostate cancer¹⁶².



Figure 4.7 (A) Elongation factor (EF) of CTCs from prostate cancer patients (in blue) compared with cultured prostate cancer cells (in grey). The median EF of CTCs was generally greater with significant interand intra-patient variability. (B) Nuclear cytoplasmic (N/C) ratios of CTCs from prostate cancer patients (in blue) compared with cultured prostate cancer cells (in grey). Median with upper and lower quartiles is shown for each sample. The median N/C ratio for CTCs was generally greater with significant inter- and intra-patient variability.

4.4.3 N/C Ratio

The nuclear cytoplasmic ratio (N/C) is defined the ratio of the apparent nuclear area and the apparent cell area with the nucleus subtracted. CTCs. As shown in Figure 4.7B, we found the median N/C for prostate cancer CTC, enriched by CellSearch[®] assay, is 1.43, which is greater than that of cultured cancer cells, 1.12 (p<0.1). This observation further stresses the potential efficacy of deformability-based CTC enrichment, as the nucleoplasm contributes 10-fold more to cell stiffness than does the cytoplasm¹⁰⁷. In addition, CTCs from CRPC patients showed significantly greater N/C ratio variability than cultured cancer cells. Considering that the N/C ratio of CTCs correlates with poor disease outcome¹⁶², it may be speculated that, within this heterogeneous population, there are cell subpopulations with greater metastatic potential. If so, then perhaps a more relevant measure of disease status is the count of a certain subpopulation of CTCs rather than the count of all CTCs as used currently^{49,176}.

4.4.4 Cell Shrinkage

One of the potential concerns associated with measuring cell size using the CellSearch[®] system is whether storing cell samples in the CellSave[®] tubes modifies the size of the cell and nucleus. To investigate, we compared cultured cancer cells spiked into blood from healthy donors processed immediately with the same cells processed after 48 hours of storage. The cell diameter was found to decrease by ~6%, while the nuclear diameter was found to decrease by ~10% from 0 to 48 hours (Figure 4.8). This result gives a sense of the variability of the measured cell morphology parameters resulting from sample storage time, but cannot explain the significant differences in morphological parameters between CTCs and cultured cancer cells or within each CTC sample.



Figure 4.8 Changes in cell and nucleus after two days of storage in the CellSave[®] tubes. The diameter of cultured prostate cancer cells decreased ~6% on average while the nuclear diameter of cultured prostate cancer cells decreased ~10% on average.

4.5 Summary

In summary, CTCs from CRPC patients, enriched using CellSearch[®] assay, were smaller in size, more elongated in shape, and had greater N/C ratio when compared to cultured prostate cancer cells. Furthermore, CTCs exhibited significantly greater viability in shape and N/C ratio. While

the system only captures EpCAM-high cells, CTC images from CellSearch[®] enumeration are widely available and can reveal morphological characteristics that are common among CTCs. The morphological differences between cultured cell lines and CTC need to be considered in the design and testing of devices that isolate CTC in a label-free fashion based on cytomorphological criteria.

Chapter 5: Development of CTC Separation Process and Analytical Pipeline

5.1 Introduction

In the previous chapter, the morphology of CTCs from prostate cancer patients and prostate cancer cell lines was investigated to assess whether the latter is an appropriate model for the former in a label-free cell separation device development. Using our customized software tool, images from CellSearch[®] system were extracted and analyzed to compare the morphological characteristics between CTCs from prostate cancer patients and prostate cancer cell lines. In contrast with cultured cancer cells, prostate cancer CTCs enriched by the CellSearch[®] system were found to have significantly smaller size, larger nuclear-cytoplasmic ratio, and more elongated shape. These CTCs were also found to exhibit significantly more viability than cultured cancer cells in nuclear-cytoplasmic ratio and shape profile. These result suggests that size of CTCs from prostate cancer patients are relatively smaller and thus there is a significant size overlap between prostate CTCs and leukocytes. This represents a significant challenge to existing label-free enrichment strategies, which sort cells by size alone. While these methods are validated by demonstrating selective enrichment of cultured tumor cells from blood, they would likely miss potential CTCs, whose size might overlap with that of leukocytes of similar size.

One approach to improve the selectivity of size-based separation methods is to distinguish CTCs from leukocytes based on cell deformability. The potential for deformability-based separation is rooted in the greater nuclear-cytoplasmic ratio (N:C) observed in CTCs compared to leukocytes^{106,110}. In fact, nuclei behaved as viscoelastic solid materials similar to the cytoplasm, but were 3–4 times stiffer and nearly twice as viscous as the cytoplasm¹⁷⁷. Since the viscosity of the cell nucleus is greater than the cytoplasm, CTCs are likely to be at least 2.5-3X more rigid

than monocytes and lymphocytes. Deformability-based separation typically involves microfiltration of live cells through an array of micro-scale constrictions. Notable examples include microfilters made using perforated plastic tape¹⁷⁸, polycarbonate membranes^{105,115}, and photholithographically fabricated microstructures¹⁷⁹.

A key challenge in the filtration of live cells is clogging, where cells caught in the filter microstructure block the path for incoming cells. The presence of these trapped cells significantly degrades the selectivity of the separation process because they induce unpredictable variations in the filter resistance. Since the deformation force applied to each cell in the filter microstructure is determined by the fluid flow rate, variations in the filter resistance leads to variations in the force applied to each cell. Additionally, prolonged contact between the cells and microstructures greatly increases the chance for adsorption and thereby limit the ability to extract the separated cell for further analysis.

This chapter presents a method for deformability-based separation of live cells that overcomes these key challenges by using the microfluidic ratchet mechanism to enable deformability-dependent transport while limiting contact between cells and the filter microstructure. Section 5.2 introduces the microfluidic ratchet mechanism and cell separation using microfluidic ratchets. Section 5.3 presents the design of the microfluidic ratchet device, followed by the details of experimental procedures in Section 5.4. Device characterization and validation is presented in Section 5.5. The optimization of the cell separation system for higher throughput is presented in Section 5.6. Section 5.7 presents the development of CTC identification pipeline following microfluidic enrichment. The chapter concludes with a summary in Section 5.8.

5.2 Approach

5.2.1 Microfluidic Ratchet Mechanism

The microfluidic ratchet mechanism previously demonstrated by Guo, Q. et al. exploits the deformation of individual cells through microscale funnel constrictions¹²³. The principle of the microfluidic ratchet mechanism involves the deformation of single cells through funnel-shaped constrictions where the opening of the constriction is smaller than the diameter of the cell. Force required to deform cells along the direction of the funnel is less than that against the direction of the funnel (Figure 5.1A and B). Therefore, oscillatory flow of an appropriate magnitude can cause cells of a specific size and deformability (or more precisely, squeezability) to "ratchet" through the funnel. Deformability-based cell separation using such a microstructure can be achieved where smaller and more deformable cells (i.e. CTCs) are blocked by the funnel and then released with each flow reversal. The combination of the oscillatory flow and the ratchet effect enables perpetual reuse of these filtration microstructures by minimizing contact between cells and microstructures to preserve selectivity by eliminating clogging and adsorption.



Figure 5.1 Principle of microfluidic ratchet mechanism. Larger and less deformable cells are prevented from transiting through funnel constitutions in forward flow (indicated by red arrow, A). Smaller and more deformable cells are unable to return through funnel constrictions under reverse flow (indicated by blue arrow, B)

5.2.2 Cell Separation Using Microfluidic Ratchets

Using the ratchet mechanism, Sarah McFaul from our group, designed a physical cell separation device (version 1.0) for batch processing of samples. The separation process, involves initially infusing a batch of cells below the first row of a matrix of funnel constrictions, which consists of 12 rows and 128 columns of microscale funnels. The pore size in each row is constant but the pore sizes between rows are incrementally smaller from the bottom to the top. Second, the cells are subjected to oscillatory pressure, wherein the forward pressure sorts the cells based on their size deformability and the reverse pressure dislodges and cells that are too large or rigid to transit the microscale funnels. During the oscillatory flow, the cells with greater deformability will transit to top rows while the cells with lesser deformability will be retained in bottom rows in the funnel matrix. Once the batch of cells has been sorted within the matrix into two cell fractions, on the basis of deformability, these fractions are separately collected. Subsequently, another batch of cells can be infused into the device and the cycle can be repeated until a sufficient number of target cells has been acquired.

Using membrane microvalves, the oscillatory flow within the funnel matrix can be precisely controlled and the system has achieved greater selectivity, when compared to other sorting methods. However, a key limitation to this initial design was that the cells are sorted in discrete batches and the system therefore has an extremely low sample throughput (less than 10,000 nucleated cells per hour).

5.2.3 Principles of Continuous Filtration Using Microfluidic Ratchet

To separate a larger number of cells, a continuous-flow cell sorting device (version 2.0) was developed with Chao Jin. The central sorting area comprises of a 2D funnel array where the funnel openings gradually decrease in size in successive rows from bottom to the top. Microchannels lining the top and bottom of the funnel array provide a biased oscillatory flow, while microchannels lining the left of the funnel array provides a constant right flow. Cells are introduced from the bottom-left of the funnel array and are pushed by a constant rightward flow while simultaneously oscillated up and down. In this manner, cells travel in a zigzag diagonal path until reaching a limiting funnel row, where they travel horizontally towards the outlet reservoirs (Figure 5.2). Cells do not experience significant deformation until nearing their blocking funnel row, where the ratchet effect permits them to only transit unidirectionally. After



Figure 5.2 Continuous deformability-based cell separation using oscillatory diagonal flow through a matrix of funnel constrictions. The blood sample infused into the bottom-left of the funnel matrix travels in a diagonal path until reaching a limiting funnel size. The path of the less deformable CTCs (shown in purple) flattens at a larger funnel row than the more deformable leukocytes (shown in blue).

reaching the blocking funnel row, the fractionated cells are constrained vertically between two funnel rows, enabling the cells to be extracted using a constant rightward flow. Red blood cells (RBCs) are not constrained by the funnel constrictions because of their extreme deformability. Consequently, they flow the bulk fluid and flow diagonally into the microchannels lining the top of the funnel array.

The combination of oscillatory flow and asymmetrical deformation enables perpetual reuse of the filtration microstructures to perform a continuous separation process. Since the cells come into contact with the filtration microstructures only momentarily, clogging and adsorption is effectively eliminated even for high-density samples like whole blood. Additionally, because target cells are not trapped by the filter microstructure during the separation process, the hydrodynamic resistance of the filter remains constant, which allows all incoming cells to experience a constant filtration force.

5.3 Design of the Microfluidic Ratchet Device

5.3.1 Tapered Constriction Design and Modeling

The tapered microstructures are designed such that a cell is laterally constrained and must deform in order to transit, but is vertically unconstrained to provide stress relief. These capabilities are essential for preventing cells from rupturing as they are deformed. For blood cells and cancer cells, a thickness (*H*) of 30 μ m or greater was experimentally determined to be sufficient (Figure 5.3A). The pore size (*W*₀) is defined as the opening of the constriction, which varies from 2-18 μ m in the CTC separation device. The pressure required to deform cells through each constriction depends on a combination of the pore size and the shape of the funnel taper,



Figure 5.3 (A) Structure of the tapered constriction determined by the parameters of microchannel thickness (H₀), pore size (W₀), and funnel taper shape (f(x)). (B,C) Modeling single cell as liquid drop to estimate its deformation pressure along and against direction of taper.

which also provides the directional asymmetry responsible for the microfluidic ratchet effect. A simple method to model the deformation of single cells through a constriction is to consider the cell as an idealized liquid drop held together by a surface tension T_0 . Since single cells are vertically unconstrained, the deformation pressure required to push such an ideal liquid drop through a constriction can be determined using Laplace's law:

$$\Delta P = T_0 \left(\frac{1}{R_a} - \frac{1}{R_b} \right) \tag{5.1}$$

where ΔP is the pressure difference required for a cell to transit through the constriction, while R_a and R_b are effective leading edge and trailing edge of the cell. When the cell transits the tapered constriction in the forward direction, both the leading edge and the trailing edge are constrained, which reduces the difference between R_a and R_b , resulting in which results in smaller transiting pressure (Figure 5.3B). Conversely, when the cell transits the tapered

constriction in the reverse direction, only the leading edge is constrained, while the trailing edge is unconstrained, which enlarges the results in a larger difference between R_a and R_b , and resulting in greater transiting pressure (Figure 5.3C). This physical asymmetry allows unidirectional ratchet transport through the constriction in the presence of oscillatory flow. By infusing cells into a sorting matrix composed of tapered funnel constrictions of incrementally decreasing pore size, cells can be fractionated under oscillatory flow on the basis of their size and deformability.

The funnel shape was previously optimized by Q. Guo et al. in our group¹⁶⁴,. The deformation pressure asymmetry as a function of the funnel shape were investigated in two forms, straight and curved tapers. The greater asymmetry was found with more abrupt tapers than more gradual tapers. However, gradual tapers also applied significantly greater compression to each cell, which greatly increased the potential for rupture. In this design, we chose a curved taper with parabolic profile, $f(x) = kx^2 + W_0/2$, where $k=2000 \text{ m}^{-1}$ because it allows more gradual deformation of the cells as they enter the funnel than straight funnel while maintaining a high degree of pressure asymmetry. A curved funnel taper has an additional benefit of avoiding the occurrence of sharp corners, which can be challenging to replicate reliably using photolithography process.

5.3.2 Continuous-Flow Microfluidic Device Design for CTC Separation

The continuous-flow microfluidic device (version 2.0) is composed of a central sorting region and supporting channels including inlets, outlets, and oscillations microchannels for flow control. The central sorting region contains a 2D array of tapered constrictions, which consist of 32 rows by 2048 columns of funnel constrictions. The opening of the funnel constrictions is consistent in each row and gradually reduced from the bottom row to the top row, ranging from 18 µm to 2 µm. Supporting microchannels lining the left, top, and bottom of the funnel array introduce precisely controlled flows into the array. Cells are infused from the bottom-left of the funnel array and experienced constant rightward flow as well as vertical oscillation. The magnitudes of the upward and downward flow are the same, but the upward flow is applied for a greater length of time to give the cells a net upward displacement. In this manner, cells travel diagonally through the funnel array in a step-wise path, until reaching a limiting funnel row where they travel horizontally towards the outlet reservoir with each cell type achieving a characteristic distribution in the funnel array. It is important to note that the cells do not experience significant deformation until nearing their blocking funnel row, where the ratchet effect permit them to only transit unidirectionally. This property constrains the cells vertically between two funnel rows, enabling the cells to be extracted using a constant rightward flow.

When whole blood is infused, the red blood cells flow to the top of the matrix and exit though the top of oscillation channel because they are not constrained by the funnel constriction. The leukocytes and CTCs travel until reaching their limiting funnel size where they proceed rightward to two different outlets. CTCs and leukocytes are specifically discriminated across several row of 6 μ m constrictions, serving as a cutoff for separating CTCs from leukocytes. Cells that can deform through these cut-off rows are collected in the leukocytes outlet while cells that fail to deform past the cut-off rows are collected in the CTC outlet. The threshold deformation pressure at the cut-off row (ΔP_{cutoff}) can be estimated from the total flow rate through the cut-off constriction row (Q) and their hydrodynamic resistance (R_{cutoff}) using the equation:

$$\Delta P_{cutoff} = Q \times R_{cutoff} \tag{5.2}$$

Oscillatory flow through the cut-off row is generated using pressure-driven flow originating from the oscillation flow channels. Similarly, the constant buffer flow orthogonal to the oscillatory flow is generated using pressure-driven flow originating from the buffer and sample inlets. To maintain a constant pressure difference across the cut-off funnel, the hydrodynamic resistance of all inlet microchannel was design to overwhelm the hydrodynamic resistance of the funnel matrix. The specific selectivity for capturing tumor cells can be adjusted from the pressure applied at the oscillation inlet, while the path taken by the cell sample through the constriction matrix can be adjusted from the relative pressure at the oscillation and buffer inlets. The throughput for processing whole blood is approximately 1 mL per hour and can vary depending on the viscosity of the blood and the pressure applied at the sample, buffer, and oscillation inlets. Optimization of these parameters were conducted together with Chao Jin during her master's thesis.

5.4 Experimental Section

5.4.1 Microfluidic Device Fabrication

Microfluidic devices were fabricated by Chao Jin using the standard photolithography. Patterns for the microfluidic ratchet device is first fabricated on a silicon wafer with two layers. The microstructures patterns were designed using DraftSight (Dassault Systems, France), and translated onto optical photomasks. One high-resolution photomask was used to generate the microscale funnel constrictions while one low-resolution photomask was used for the flow channels. In the first layer, the microstructures in the central sorting region are fabricated using

negative photoresist SU-8 8025 (Microchem, MA). The silicon wafer was coated with negative photoresist SU-8 8025, spun at 3000 rpm for 30 sec to produce a 30 μ m thick layer. The wafer was then baked at 95 °C for 5 min and exposed to UV light though the optical photomask, followed by post-bake steps at 65 °C (1 min), 95 °C (4 min), and 65 °C (1 min), and washed with isopropanol. The second layer formed the supporting flow channels and aligned with the first pattern. The second set of SU-8 features patterned as described above, with a spin speed of 2200 rpm for 30 sec to create the height of this layer as 40 μ m. The structures were hardened by ramping the temperature from 40 °C to 165 °C, at increments of 15 °C/10 min. The silicon wafer was then incubated at 165 °C for 30 min and cooled to 65 °C by ramping the temperature by 50 °C/10 min. The final heights of the first and second layer were measured to be 29.6 and 40.1 μ m, respectively.

5.4.2 **PDMS Device Fabrication**

The replicas of the silicon wafer molds were fabricated using soft-lithography of Sylgard 184 polydimethylsiloxane (PDMS) silicone. The silicon wafer with microstructures was placed on a 15-cm diameter Petri dish, and secured in the center with the tape. PDMS mixture (base:curing agent = 10:1) was poured into the petri dish to a thickness of ~5 mm, and the petri dish was placed in a vacuum chamber for 15 min to void air bubbles from the features. The petri dish was subsequently baked at 65 °C for 2 hours to cure the PDMS. Cured PDMS was gently peeled off from the silicon wafer, and holes were made using a 0.5 or 6 mm hole punch (Harris, CA). PDMS layer is attached to a glass slide by 90 sec of activation in air plasma (Harrick Plasma, NY), followed by baking the PDMS devices at 65 °C for 10 min. All PDMS devices were cooled to the room temperature before using them.

5.4.3 **Tumor Cell Doping Experiments**

The UM-UC13 bladder cancer cell line, provided by Pathology Core of the Bladder Cancer SPORE at MD Anderson Cancer Centre, was cultured in minimum essential media (MEM), supplemented with fetal bovine serum (10% v/v), 1% L-glutamine, 1% MEM nonessential amino acids, and 1% sodium pyruvate, as well as 1% penicillin-streptomycin. The cells were maintained in humidified incubator at 37 °C and 5% CO₂.

Prior to doping experiments, UM-UC13 cells were stained with Calcien AM Green and leukocytes in whole blood were stained with Hoechst 33342. Whole blood was drawn from healthy donors into 6 ml of EDTA blood collection tube. Number of UM-UC13 cells were manually counted using a hemocytometer, and doped into 5 ml of whole blood at a ratio of 1 tumor cell to 1000 leukocytes, except where indicated otherwise, and processed using the microfluidic ratchet device. After separation, the relative number of Calcien AM Green-labeled UM-UC13 versus Hoechst 33342-labeled leukocytes in both CTC and leukocytes outlets were counted to determine yield and enrichment. The parameters for yield, enrichment, and throughput are defined as follow:

$$Yield = \frac{Target \ Cells_{output}}{Target \ Cells_{input}} \times 100\%$$
(5.3)

$$Enrichment = \frac{(Target Cells/Background Cells)_{output}}{(Target Cells/Background Cells)_{input}}$$
(5.4)

Throughput =
$$\frac{Total Volume of Sample}{Total Time to Process}$$
 (5.5)

Viability of the separated UM-UC13 cells was assessed using the MTT assay both prior to and after microfluidic enrichment. The enriched cells were subsequently cultured for 10 days to assess the proliferative capacity of the cells.

5.4.4 Microfluidic Device Operation

The cell separation processes involved initially filling the microfluidic device for 15 min using buffer solution, 0.2 % Pluronic F127 (Invitrogen) in PBS, to prevent nonspecific adsorption of cells to the wall of the device. Whole blood samples were infused at the left-bottom corner (sample inlet), while buffer solutions are introduced through buffer and oscillation inlets. Pressure-driven flow originating from sample and buffer inlets was controlled using a commercial pressure controller (Fluigent, France), while flow from oscillation inlets was controlled using a custom-made pressure control system and software. Experiments were conducted under an inverted microscope (TS-100, Nikon), and a CCD camera (DS-2MBW, Nikon) to observe pre-stained cells in the microfluidic ratchet device. After separation, the device was put aside for 1 hour in dark to collect the cells at the bottom of the reservoirs before fluorescence images were acquired. In order to determine the number of pre-stained cells in outlet reservoirs, multiple fluorescent images were acquired with 4x objectives, and the images were stitched together using Microsoft Image Composite Editor. The numbers of tumor cells and white blood cells were then counted manually from the composite images. The performance metrics of yield and enrichment are calculated from the count values. For system characterization, different oscillation pressure and various doping ratio of tumor cells to leukocytes were applied to evaluate the performance of the microfluidic ratchet device.

5.5 System Characterization

5.5.1 **Device Performance**

The performance of the microfluidic ratchet device was evaluated using fluorescently labeled UM-UC13 bladder cancer cells that were doped into whole blood with labeled leukocytes. The distinct vertical displacement of leukocytes and cancer cells can be demonstrated by observing fluorescently labeled cells within the sorting area (Figure 5.4). Specifically, both leukocytes and cancer cells travel in a diagonal path until reaching their limiting funnel size, where the ratchet effect permits them to transit only unidirectionally. After reaching the blocking funnel row, the fractionated cells are constrained vertically between two funnel rows, enabling them to be extracted using a constant rightward flow. Highly deformable cells, such as red blood cells, are



Figure 5.4 (A) Photograph of the microfluidic ratchet device infused with colored water to show the diagonal flow pattern in the separation matrix. (B) Composite fluorescence image of leukocytes (blue) and doped UM-UC13 cancer cells (green) in the funnel matrix during a separation process, demonstrating the vertical displacement.

not constrained by the funnel constrictions and flow diagonally into the microchannels lining the top of the funnel array.

The yield and enrichment of tumor cells can be tuned by adjusting the pressure applied at the oscillation inlet. The pressures applied at the sample, buffer, and oscillation inlets are initially optimized experimentally by observation of the path taken by red blood cells to ensure that they follow a diagonal path within the funnel matrix. These initial pressure parameters were further



Figure 5.5 (A,B) Performance of deformability-based separation of UM-UC13 cancer cells doped into whole blood at the cancer cell to leukocyte ratio of 1000:1. Enrichment and yield are characterized as a function of deformation pressure (at the 6 µm cutoff funnels). (C,D) Relationship of doping ratio and deformabilitybased system performance. Enrichment and yield are characterized as a function of doping ratio of cancer cells to leukocytes.

optimized by separation of UM-UC13 bladder cancer cells doped into whole blood. At a doping ratio of 1 tumor cell to 1000 leukocytes, it was observed that a higher applied pressure could achieve tumor cell enrichment of 10^4 -fold (Figure 5.5A). However, while low-pressure operation had a 90% capture rate, this higher pressure only captured 70% of tumor cells (Figure 5.5B). We hypothesized that the performance of this system was limited by crowding of the row funnel pores by cancer cells at the higher doping ratios. Consistent with this hypothesis, we found that doping cells at lower and more biologically-relevant ratios, as low as 1 tumor cell per 10^5 leukocytes, permitted ~ 10^4 -fold enrichment even at lower applied pressure (30 kPa), where the yield was 94.4 \pm 1% (Figure 5.5C and D). The throughput for processing whole blood is approximately 1 mL per hour and can vary depending on the viscosity of the blood.

5.5.2 Impact of Deformability on Enrichment of Cancer Cells

Many existing microfiltration enrichment strategies require that the cells be partially fixed by formalin prior to analysis^{115,118,179,180}. This fixation contributes to rigidification of these cells and causes these enrichment strategies to sort cells on the basis of size but not deformability. Conversely, the microfluidic ratchet mechanism enriches for live unprocessed cells, allowing them to be sorted based on both size and deformability. To evaluate the impact of deformability on cell sorting, we compared our observed enrichment to the enrichment expected if the cells were to behave as immutable spheres. Under these assumptions we expected selective enrichment of tumor cells to be less efficient if these cells were equal in size, or smaller than contaminating leukocytes. We assessed 100 of each cell type and measured the mean diameter of both leukocytes and UM-UC13, $9.9 \pm 1.8 \mu m$ and $17.6 \pm 2.9 \mu m$, respectively (Figure 5.6). If the



Figure 5.6 (A) Size distribution of leukocytes and UM-UC13 cancer cells. The black lines represent fitted normal distribution for the size measurements. B) Enrichment of doped UM-UC13 cancer cells obtained through deformability based separation using the microfluidic ratchet device compared to the optimal performance obtained through size-only separation calculated from cell size distribution in A.

cell size conforms to a normal distribution and we imagine that each cell represents an immutable sphere, the theoretical enrichment for an ideal size-only separation process could be determined. By comparison with empirical results obtained using the microfluidic ratchet device, size and deformability-based separation improves enrichment by 18x to 100x at a capture rate of 77% to 90%, respectively (Figure 5.6). This is likely due to the fact that while tumor cells like CTCs may exhibit significant overlap with the size of contaminant leukocytes^{103,106,162}, the tumor cells are more rigid and thus more likely to behave as immutable spheres. Consequently, deformability-based enrichment is markedly more selective than enrichment based on cell size alone.

5.5.3 Viability and Proliferative Capacity of the Enriched Cells

While cell enrichment methods typically involve cell fixation, which renders the cells nonviable. The microfluidic ratchet mechanism can sort unfixed cells that remain intact and viable. To assert that the viability of sorted cells is not adversely impacted by oscillatory flow through the funnel matrix, we performed LIVE/DEAD staining on UM-UC13 cells, following enrichment. Cell viability following enrichment was $99.1 \pm 1.9\%$. Furthermore, UM-UC13 cells, collected from the collection outlet and cultured in a 96-well culture plate alongside unsorted cells that were seeded at a comparable cell density. Compared to unsorted cells, sorted UM-UC13 cells proliferated at a similar rate and displayed a similar increased production of metabolic products, as determined by MTT assay (Figure 5.7).



Figure 5.7 (A) Cell growth of UM-UC13 cells after cell separation. (B) Cell number increase during subsequent culture. (C) Assessment of cell metabolic activity based on MTT assay.

5.6 Optimization of CTC Separation for Higher Throughput

While the throughput of the continuous-flow microfluidic device (version 2.0) (1 mL/hr of whole blood) greatly improved over the previous batch-mode device (version 1.0), sample throughput could be further enhanced to support clinical application. In addition, downstream characterization of tumor cells, such as single cell sequencing (discussed in Chapter 6) necessitates the processing of larger volumes of patient blood. Therefore, we evaluated a range of red blood cell lysis protocols in order to pre-enrich the CTC specimen with minimal risk for target cell loss (Section 5.6.1). The use of a lysis buffer creates cell debris and clumps, which can cause the device to become clogged and eventually fail. Therefore, a new version of the device with the improved channels design (version 2.1) was developed by Justin Yan during his master thesis¹⁸¹ (Section 5.6.2). This device was tested and optimized using lysed samples together with Justin Yan (Section 5.6.3).

5.6.1 Red Blood Cell Lysis Process Selection

To increase the throughput of the cell separation process, red blood cell lysis step was introduced prior to microfluidic enrichment. Several red blood cell (RBC) lysis methods were tested using 22RV1 prostate cancer cells doped into whole blood. 22RV1 cells were pre-stained with Calcien Green and counted manually using a fluorescent microscope before and after RBC lysis process. The detailed protocols for each RBC lysis method are available in Appendix B. The recovery rate is calculated as below.

$$Recovery \ rate = \frac{Target \ Cells_{after \ RBC \ lysis}}{Target \ Cells_{before \ RBC \ lysis}} \times 100\%$$
(5.6)

A lower pre-enrichment recovery rate would result in fewer cells proceeding towards the microfluidic enrichment step, where even fewer cells could be collected at the end of the separation step. In addition, the processing time was kept to the minimum in order to allow more time for CTC enrichment and single cell isolation steps (discussed in Chapter 6) while viability of cells was maintained. Therefore, we chose the method with the optimal recovery rate and lower processing time.

By comparing the recovery rate for RBC lysis methods, we chose to use RBC lysis buffer (G-Biosciences) because it could recover 74% of target cells in only 10 min (Table 5.1). This method involves a simple 5-min incubation step with lysis buffer, followed by 5-min cell collection using the centrifugation. Compare to other pre-enrichment methods, the processing time was significantly shorter and the process was much easier and simpler and thus it was selected.

RBC Lysis Methods	Recovery Rate	Processing
		Time
EasySep with CD45 and GlyA	19.5 ± 4.95 %	50 min
Density Gradient Centrifugation with Histopaque 1119	77.99 ± 3.42 %	40 min
RosetteSep with CD45	68.75 ± 8.84 %	40 min
RBC lysis buffer (Ammonium Chloride)	61.04 ± 4.18 %	20 min
RBC lysis buffer from G-Bioscience	74.0 ± 2.83 %	10 min

Table 5.1 Comparison among various RBC lysis methods.
5.6.2 Multiplexed Inlet Microfluidic Device Design and Operation

The multiplexed inlet microfluidic device (version 2.1) was developed by Justin Yan in order to process blood samples after RBC lysis for higher throughput¹⁸¹. One of the major limitations of the previous version of microfluidic ratchet device (version 2.0) is the accumulation of cell debris at the sample inlet. The geometry of the inlet of the continuous flow device is such that it has a single entry point for incoming cells to the sorting region. As the samples are processing, cell debris accumulates, and eventually blocks the inlet channel, causing the device to fail. Furthermore, RBC lysis potentially introduces more cell debris and clumps, increasing the chance of device failure. Thus, the microchannels design in device were modified to include



Figure 5.8 (A) Representative image of the device with a multiplex sample inlet. (B) Photograph of the microfluidic device with multiple inlets shown in a red dotted rectangle.

multiple inlets to create bypass routes for cells, allowing them to flow into the sorting region if some inlet channels are blocked by potential cell debris. Specifically, the sample inlet is relocated to be in parallel with the forward inlet, and connected through a series of bifurcations to take up 1/4 of the channels on the bottom of the device, while the forward inlet occupies the remaining 3/4 (Figure 5.8). All other flow channels and the sorting matrix design were identical to the continuous flow device.

The device was operated in a similar manner with a previous version of the device. The device was pre-filled with the PBS with 0.2% Pluronic F127 (Invitrogen, Carsbad, CA) to prevent non-specific adsorption of cells in the device for 10 min. The cell sample and fluid were applied to the device through a series of fitting and tubing connecting in a 10cc syringe tube (Nordson EFD, Westlake, OH), which acts as the fluid reservoir. Fluid handling for the experiment was performed using a commercial pressure controller (Fluigent, Paris, France), and custom made pressure board and software, developed and optimized by Justin Yan.

5.6.3 Multiplexed Inlet Device Performance using Lysed Blood Sample

In order to validate the multiplexed inlet microfluidic device (version 2.1), we used pre-stained UM-UC13 bladder cancer cell lines doped into whole blood. First, we validated the multiplexed inlet microfluidic device (version 2.1) by processing the whole blood and we compared the yield to the previous version 2.0 of the device (the continuous-flow device). The mean yield of the continuous flow device for whole blood was $80.99 \pm 4.13\%$ while that of the multiplexed inlet microfluidic device (version 2.1) was 83.01 ± 1.71 using similar operating condition (Figure 5.9A). Using the student t-test (unpaired), there was no significant difference in two samples (p-



Figure 5.9 (A) Yield comparison between the continuous-flow device (version 2.0) and the multiplexed inlet microfluidic device (version 2.1). (B) Yield comparison between RBC lysis treated sample and non-treated samples.

value > 0.05). This suggests that the device performance was not altered by modifying the microchannels in inlet area. Second, we compared the yield for samples with and without RBC lysis step using the multiplexed inlet microfluidic device (version 2.1). Using UM-UC13 doped whole blood, we performed RBC lysis (G-Bioscience lysis buffer) and processed the specimen using the multiplexed inlet microfluidic device (version 2.1). Device performance was then compared to the baseline device performance without RBC lysis. In contrast to lysis-free enrichment (83.01 \pm 1.71 %), RBC lysis sample captured 78.14 \pm 2.31 % of target cells, which did not represent a significance (p>0.05; Student T-test) (Figure 5.9B). However, the throughput was increased eight-fold from 1 mL per hour to 8 mL per hour. This increase in throughput, without degradation of performance, allows for the processing of the higher volume of blood to enrich rare CTCs from prostate cancer patient samples towards single-cell analysis workflow (discussed in Chapter 6 and 7).

5.7 CTC Identification Process Development

In previous sections, a microfluidic device that separates cells based on deformability was developed, tested and optimized. Following cell separation, immunofluorescence staining was used to discriminate CTCs from contaminant leukocytes. The goal was to develop the CTC enumeration system, including the immunofluorescence staining and the spectral analysis using the Zeiss Zen laser scanning microscopy (LSM) 780 system. First, the immunofluorescence staining protocol was developed and tested using the prostate cultured cancer cell lines (Section 5.7.1). Next, the samples were scanned and analyzed using spectral data from images by the Zeiss LSM 780 system (Section 5.7.2). The optimized protocol is used for CTC enumeration in patient samples in Chapter 7.

5.7.1 Immunofluorescences Staining for CTC Identification

Following enrichment, immunofluorescence staining is typically used to discriminate CTCs from hematological cells, based on the presence of epithelial (e.g. cytokeratin, EpCAM) antigens and/or tumor-specific antigens such as AR or PSMA in prostate cancer^{182,183} as well as the absence of hematological antigens (e.g. CD45). Here, we adopt the general immunofluorescence protocol to identify target tumor cells in our samples. Specifically, our goal was to use the conventional definition of a CTC, defined as a nucleated CK+/CD45- cell, by immunofluorescence staining⁷⁸. Cytokeratin (CK) is a family of at least 29 different cytoplasmic structural proteins in almost all cells of epithelial origin, and thus its antibody is broadly used in detection for CTCs derived from epithelial tumors (e.g. breast, prostate, colon, and lung). Conversely, CD45 is a membrane protein, that is expressed on all leucocytes¹⁸⁴. In addition to CK and CD45, EpCAM and/or AR antibodies are added to the staining process for further



Figure 5.10 LNCaP simultaneously stained with for CK, AR, EpCAM, CD45 and DAPI. (B) Leukocytes stained negative for epithelial (EpCAM and CK) and prostate tumor (AR) antigens but positive for CD45.

characterization of target tumor cells. EpCAM is the most commonly used epithelial cells surface maker in CTC enrichment using affinity-based methods such as the CellSearch[®] system, while AR is a prostate-specific marker localized in the nucleus. The prostate cancer cell lines (e.g. LNCaP) were used in testing of antibodies. The list of antibodies used in this protocol is available in Appendix C.

In order to achieve comprehensive antigen profiling of CTCs, we optimized an immunofluorescence protocol to simultaneously immunostain for CD45, CK, EpCAM, and AR, while staining the nucleus with DAPI. The primary challenge to development of this immunostaining assay was the potential for overlap between fluorophore spectra. We therefore selected Alexa Fluor[™] fluorescence antibody conjugates because their emission spectra span

both the visible and infrared range. By combining the conjugates CK-Alexa 488, AR-Alexa 550, EpCAM-Alexa 594 and CD45 conjugated to Allophycocyanin (APC), we were able to discriminate each antigen biomarker with minimal spectral overlap. Briefly, this assay used LNCaP prostate tumor cells grown to 70% confluence on a cover slip that were fixed (4% formaldehyde, 15 min), washed twice (PBS, 5 min), permeabilized (0.025% of Tween-20, 10 min) and washed twice more. For immunostaining, the samples were blocked (3% bovine serum albumin in PBS, 1 hour at room temperature) and incubated with the antibody cocktail at 4 °C overnight. The specimens were DAPI stained immediately prior to scanning using the Zeiss LSM 780 system. Following optimization, 0.05 µg for CK-, 0.1 µg for EpCAM-, and 1 µg for AR-specific antibodies were found to be optimal in 100 µl staining volume, enabling us to detect epithelial (CK, EpCAM) and prostate tumor (AR) biomarkers specifically in LNCaP prostate tumor cells, and the CD45 leukocyte antigen was absent (Figure 5.10A).

After optimization of the antibody concentrations in the staining cocktail, a secondary challenge was to stain the cells in suspension and visualized them in a 384-well imaging plate. This was a critical step because both leukocytes and CTCs are obtained in suspension, following microfluidic ratchet enrichment. To optimize leukocyte staining, leukocytes were extracted from whole blood using the density gradient centrifugation and stained as described above but instead in suspension, in a 1.5 ml minicentrifuge tube. Washing and buffer exchange was conducted following centrifugation at 200 xg for 5 min. Then, stained leukocytes were transferred to 384 optically transparent well plate (Corning) for microscopy scanning process. The 384 well plate has the optically clear flat well bottom, allowing the direct microscopic viewing using the Zeiss 780 microscope system without using the coverslip. The well plate was centrifuged at 200 xg for 5 min.



Figure 5.11 LNCaP identified as DAPI+CK+EpCAM+CD45- and leukocytes identified as DAPI+CD45+.

5min before scanning with the Zeiss LSM 780 microscope. Using this protocol, leukocytes were successfully stained with CD45-APC and scanned on the 384 optically transparent well plate (Figure 5.10B). The optimal amount of CD45 antibody was determined to be 0.027 μ g in 100 μ l per up to 5 x 10⁶ cells/mL.

Finally, LNCaP prostate cancer cells were doped into whole blood, and separated using the microfluidic device. Collected samples in the CTC outlet was stained with CK, EpCAM, and CD45 in suspension using the protocol above. Stained cells were transferred to the 384 well plate, counterstained with DAPI, and scanned using the Zeiss LSM 780 microscope. As shown in Figure 5.11, both enriched LNCaP cells and contaminant leukocytes were effectively stained, implying that the developed protocol can be used with the samples in suspension after microfluidic enrichment. The LNCaP cells could be distinguished from leukocytes due to the absence of CK (shown in green) staining on leukocytes. Unlike the samples grown on the cover slip, the staining for EpCAM was not so clear on LNCaP cells stained in suspension. Furthermore, some potential CK+ leukocytes (leukocyte2; white dotted line in figure 5.11), or potential low CK+ LNCaPs with high EpCAM expression were observed, which complicates the

identification process using the visual inspection. This could negatively impact on CTC enumeration process as the false-positive rate might increase due to misinterpretation of images. Realizing the limitations of visual inspection, the spectral image analysis was employed to develop a sensitive CTC enumeration protocol.

5.7.2 Spectral Image Analysis

Using the Zeiss LSM 780 imaging system, a sensitive CTC enumeration protocol that identified CTCs by multispectral analysis of individual cells, stained with DAPI, CK, EpCAM, and CD45 was developed together with Richard Ang in our group. The multispectral approach collects spectroscopic information for each pixel of an image as a spectrum and can be used to quantify the degree to which a specific wavelength contributes to that spectrum. For this reason, fluorescence signals at different wavelengths can be unmixed to permit sensitive distinction between fluorescence biomarkers. Furthermore, high-energy excitation by conventional



Figure 5.12 Emission spectra of Alexa 488, Alexa 555, Alexa 594, and APC, demonstrating its own distinct peak. Solid lines represent the emission wavelengths for each fluorochromes, while dotted lines represent the excitation wavelengths. [Image generated from Fluorescence SpectraViewer, ThermoFisher]

fluorescence microscopy systems, not only increases the fluorescence signal, but also the autofluorescence signal that may arise due to cell and apoptotic debris. Conversely, multispectral imaging provides superior detection of CTC signal with a high signal-to-noise ratio.

In the process of CTC enumeration, the samples in the 384 well plate were scanned under a 40x oil immersion objective over 15x15 frames using a motorized stage to scan the entire well area. Each captured image consisted of a 34 color spectral image including a transmission-photomultiplier (T-PMP) channel (bright field), a low-wavelength PMT channel (DAPI), and 32 channels captured using a GaAsP spectral detector (CK/EpCAM/CD45). Antibodies used in CTC identification are conjugated with different fluorophores, which have distinct emission wavelengths (Figure 5.12). Thus, cells can be identified based on the fluorescence maxima for each fluorophore. Specifically, CTCs can be identified by a clear peak at 521 nm (CK-Alexa488) without a peak at 660 nm (CD45-APC). Meanwhile leukocytes can be classified with a spectral maximum at 660 nm (CD45-APC). In a doping experiment, enriched LNCaP cells showed a clear peak at 512 nm (CK-Alexa488) and 618 nm (EpCAM-Alexa590) without a peak at 660 nm (CD45-APC). This spectral analysis is a more objective method to identify the target cells because it is based



Figure 5.13 Fluorescence microscopy image and corresponding spectra for LNCaP (DAPI+CK+EpCAM+CD45-) and leukocytes (DAPI+CD45+).

on the spectral information, not just on the displayed colors. The CTC enumeration system using the spectral analysis is further optimized to automate the process using the customized software (discussed in Section 6.4).

5.8 Discussion and Summary

Conventional microfiltration strategies are limited because they are prone to clogging and cannot enrich for cells at high flow rate without first chemically fixing them. The result is that these systems only sort for the target cell on the basis of size only and do not capture viable cells that are easily extracted from the sorting matrix. The continuous flow microfluidic ratchet system overcomes these limitations by sorting cells based on deformation through tapered constrictions under oscillating flow, allowing the enrichment of CTCs from whole blood at a rate of 1 ml per hour. Oscillating flow through incrementally smaller microstructures ensures that the device can be operated perpetually without clogging and the enriched cells are directed into a collection reservoir where they are viable and available for downstream characterization. This mechanism allows for enrichment of tumor cells at a factor of 10^4 and a capture efficiency of ~94%. The ability to separate CTCs based on deformability and size, rather than solely based on size, is especially important in prostate cancer where CTCs are known to be smaller in size than CTCs from breast and lung cancer^{103,106,130}. Specifically, CTCs from prostate cancer has been estimated to be 8-11 μ m in diameter, which is likely to overlap with a significant fraction of the leukocyte population^{103,106,185}. Furthermore, larger hematological cells, such as large monocytes and megakaryocytes may also be difficult to distinguish from CTCs based on size alone ¹⁸⁵. However, leukocytes are significantly more deformable than tumor cells¹⁰⁹ and even larger leukocytes may be discriminated on the combined basis of size and deformability. Using cell deformability as an additional discriminating factor enables leukocytes with similar size, but with different deformability, to be separated. This additional separation criterion increases the selectivity of the separation by 10-100 fold to achieve an enrichment of ~10⁴ when tested using cultured cancer cells.

In summary, a deformability-based cell separation using the microfluidic ratchet mechanism is developed to separate live CTCs from whole blood. The microfluidic ratchet device captures >90% of cancer cells from whole blood to achieve 10⁴-fold enrichment of target cells, over contaminating leukocytes at the rate of 1 mL of whole blood per hour. The enriched cells were ~99% live and demonstrated a great proliferation capacity. The CTC separation process was further optimized to include the red blood cell lysis step for higher throughput. In turn, the multiplexed inlet microfluidic device was developed to process the samples treated with RBC lysis buffer, allowing for a significant increase in throughput from 1 mL to 8 mL per hour. In addition, an analytical pipeline that identifies the target cells using the multispectrum analysis

was established. This approach provides a more objective method to identify CTCs as it is based on the quantitative spectral data. By combining microfluidic ratchet enrichment and multispectrum analysis, this system permits CTC enrichment from clinical patients for various downstream processes (Chapter 7).

Chapter 6: Development of Single Cell Isolation and Analysis Workflow

6.1 Introduction

In the previous chapter, a label-free CTC separation device that enrich viable cells based on their distinct deformability relative to leukocytes was developed. This mechanism leverages the deformation of single cells through tapered constrictions using oscillatory flow in order to generate a ratcheting effect that produces distinct paths for CTCs, leukocytes, and red blood cells. In doping experiments, this microfluidic device captures >90 % cancer cells and enriches the concentration of tumor cells by $\sim 10^4$ -fold. While maintaining its capability, the cell separation process was further optimized to process the higher volume of the blood to increase the chance of detecting rare CTCs. Although we were able to significantly increase the throughput, the enriched samples were not pure enough for direct CTC genotyping due to the overwhelming contaminant leukocytes. In bulk analysis, key mutations that might be relevant in clinical decision could be easily obscured by contaminants leukocytes or CTC heterogeneity. Thus, the presence of contaminants, along with the potential heterogeneity of CTCs, suggests the need to develop methods to isolate and sequence single CTCs.

While significant advances have been made in single cell sequencing technology, the isolation of rare cells remains technically challenging. Micromanipulation is most commonly used to isolate and sequence single CTCs in recent single CTC sequencing studies^{143,146}. Although this method is simple and convenient, it has been criticized for being laborious and time consuming for routine clinical application. Alternative method, flow cytometry has enabled the high throughput single-cell isolation (50,000 cells/sec)¹⁸⁶. However, flow cytometry requires a minimum ~10⁵ input cells, containing more than 0.1% target cells, and is therefore incompatible with the

isolation of exceedingly rare CTCs^{143,146}. Recently, the DEPArray platform has been developed to isolate single cells using dielectrophoresis with image cytometry criteria with highly sensitive CTC capture^{142,187,188}. However, high cell loss (>60%) during enrichment and single cell isolation is a critical drawback in the case of rare-cell isolation^{188,189}. Consequently, there still a critical need for technologies that can efficiently isolate individual CTCs.

Laser capture microscopy (LCM) is a compelling single-cell isolation process because, like micromanipulation, single cells can be precisely selected and cell capture can be visually confirmed. Due to the precision of this system, the LCM is extensively used in tissue samples that are typically fixed and embedded in paraffin wax on a glass slide. However, a significant cell loss has been reported when applied to the samples from cytology sources (e.g. cytospin and conventional smear)¹⁹⁰. This cell loss arises either by target cell adsorption to the cytofunnel, during centrifugation, or during the intensive fixation and washing process. Given this propensity to lose the majority of target cell (75%) at low cell number (<300 cells)¹⁹⁰, this is a critical limitation when studying rare cells, such as CTCs.

Recently, LCM has been widely employed for genetic characterization of single cells combined with pre-enrichment of cells using microfiltration or microfluidic devices ^{191–193}. For example, pre-enriched CTCs using the ScreenCell microfiltration system has been isolated individually using the LCM method for single cell sequencing¹⁹². Although ScreenCell is highly sensitive, it lacks selectivity and single cells may be obscured within aggregates¹⁹². Furthermore, formaldehyde fixation used in this procedure crosslinks DNA and reduces the efficiency of downstream genotyping^{194,195}. The another approach used NanoVelcro chip to capture CTCs on

the functionalized nanofiber substrate, and isolate single CTCs using the laser cutting¹⁵³. This method is more selective but employs methanol fixation, which might contribute to the DNA damage, as only the small portion of isolated CTCs yielded DNA of sufficient quality for subsequent genotyping.

In this chapter, a single-cell isolation and sequence workflow has been developed that can be combined with our label-free CTC separation technology (discussed in Chapter 5). The rest of chapters are organized as follows: Section 6.2 describes overview of the single-cell isolation and analysis workflow. Section 6.3 includes the details of experimental protocol. Section 6.4 presents the development of single-cell isolation process using a hydrogel matrix and laser capture microdissection (LCM) as well as an automated CTC enumeration process. Section 6.5 includes the validation of the single-cell analysis workflow using cultured cancer cells doped into whole blood, followed by the summary in Section 6.6.

6.2 Approach

In the previous chapter, a label-free microfluidic cell separation device was developed to enrich CTCs directly from whole blood. This microfluidic device is able to capture >90% of cancer cells from unprocessed whole blood to achieve 10⁴-fold enrichment of target cells relative to leukocytes. Here, a single cell isolation and analysis workflow was developed using laser capture microdissection (LCM) for genome sequencing that can be combined with our label-free CTC enrichment method. After microfluidic enrichment, cells were live-stained to minimize potential DNA damage and ensure the compatibility with downstream Whole genome amplification (WGA). Live-stained samples were then encapsulated within a hydrogel, creating an artificial

'tissue-like' substrate for single-cell extraction using LCM. Furthermore, target cells to be isolated were identified using an automated CTC enumeration software, developed by Richard Ang in our group, to create an efficient workflow. Target cells in a hydrogel layer were then extracted using the Zeiss PALM LCM system, and further subjected to whole genome amplification step as well as characterization process including qPCR, Sanger sequencing, and targeted next generation sequencing. Finally, the entire single-cell isolation and analysis workflow is tested using cultured cancer cells doped into whole blood, demonstrating its potential to apply in the clinical samples.

6.3 Experimental Section

6.3.1 Cell Lines and Sample Preparation

Initial process development for sample encapsulation and isolation employed two bladder cancer cell lines, UM-UC13 and UM-UC3, maintained as described previously (Section 5.4.3). UM-UC13 cell line was stably transduced with enhanced green fluorescence protein (EGFP), while UM-UC3 cell line was stably transduced with red fluorescent protein (mCherry) to simplify the single-cell isolation development process. Before the experiment, cells were treated with Trypsin-EDTA (0.25%) (Thermo Fisher Scientific) for less than 1 min and washed twice with PBS. If necessary, cell number was counted using an automated cell counter (TC20, Bio-Rad). For doping experiments, two prostate cancer cell lines, LNCaP and 22RV1 were spiked into the whole blood. Cancer cells were doped before the red blood cell lysis step (described in Section 5.6.1 and Appendix B) for a single cell analysis workflow. The blood was collected in a 5 ml of EDTA tubes (BD, Franklin Lakes, NJ) from healthy donors.

6.3.2 Identification of Tumor Cells using Fixation-Free Immunofluorescence

Tumor cells were identified by fixation-free live-cell staining. Enriched cells were blocked in 3% bovine serum albumin (BSA), in PBS, for 30 min at room temperature and stained with Alexa 488-conjugated anti-EpCAM (CellSignaling), APC-conjugated anti-CD45 (Biolegend), and Hoechst 33342 (Invitrogen) for 2 hours at room temperature. Subsequently, cells were washed with PBS, deposited in the reservoirs of the PDMS baffle (radius=3mm) on a glass slide and briefly centrifuged before scanning with a Zeiss LSM 780 imaging system. The entire well was imaged under a 40x long distance objective over 15 x 15 frames using a motorized stage. Each capture image consists of a 34 color spectral image including a low-wavelength PMT channel (Hoechst), 32 color channels captured using a GaAsP spectral detector (EpCAM/CD45), and a transmission-photomultiplier (T-PMT). Scanned images were processed using our customized software to identify CTC candidates (will be discussed in Section 6.4.4). This software isolated single cell images and ranked each putative CTC. Individual cells were manually reviewed and candidate CTCs were selected for extraction. Positional information of each selected cell was collected and used for cell localization and extraction by the PALM MicroBeam laser microdissection system (Carl Zeiss, Thornwood, NY).

6.3.3 Cell Encapsulation using a Hydrogel Matrix

To encapsulate the enriched cells in a hydrogel matrix, we first infused the cell sample into a reservoir of a PDMS baffle on a glass slide (Figure 6.1). The PDMS baffle with 6 mm deep reservoirs was manufactured using a stereolithography (SLA) 3D printed mould (Objet30, Stratasys, Eden Prairie, MN). Moulds featured 3 mm diameter pillars arranged in a 2 x 3 matrix with a 7 mm intrapillar spacing. The PDMS baffle was subsequently fabricated using a standard



Figure 6.1 PDMS baffle. It allows the concentration and deposition of cells on a glass slide using centrifugation.

soft lithography technique. The reservoirs in the PDMS baffle are designed to have a diameter of 3 mm, which is similar to the conventional 384-well plate for easier handling and scanning process. After the microfluidic enrichment, cell suspensions were introduced into each reservoirs of the PDMS baffle, followed by overlaying the polyethylene glycol diacrylate (PEGDA) solution, which forms a hydrogel after crosslinking by exposure to UV light. After curing, unpolymerized PEGDA was removed by pipetting and replaced with 15 µl DNase-free water to prevent desiccation. Cells encapsulated in the hydrogel were identified by fluorescence microscopy and single cells were extracted, using the PALM MicroBeam laser microdissection system.

6.3.4 Single Cell Extraction Using LCM

Target cells in the PEGDA layer were laser microdissected following the manufacturer's protocol for the PALM MicroBeam LCM System (Zeiss). To enable the LCM extraction, we prepared samples on a polyethylene naphtalate (PEN)-membrane glass slide (PEN MembraneSlide). The PEN MembraneSlide consists of 2 µm-thick PEN membrane that is easily cut together with the sample and acts as a stabilizing backbone during lifting. After identifying a target cell, a circle line was drawn around the target cell, and a dot was placed inside an area of interest without touching the target cell. Then, the area of the interest is cut along a defined line

using a fine focused laser beam, and catapulted in an AdhesiveCap of the collection tube. The AdhesiveCap provides the instant immobilization of the catapulted sample as well as prevents the drying of the sample during collection process. After the catapulting process, we inspected the sample collected in the AdhesiveCap using CapCheck function. The Capcheck function automatically moves the stage to focus and locate catapulted items on the AdhesiveCap of the collection tube. After visual confirmation, the collection tube is carefully dismounted from the microscope, keeping the up-side down position for further whole genome amplification process.

6.3.5 Whole Genome Amplification (WGA) of Extracted Single Cells

After single cell extraction, WGA was carried out to extract and amplify genomic DNA using the REPLI-g Single Cell Whole Genome Amplification kit (Qiagen) according to the manufacturer's protocol. Briefly, 4 µl of PBS and 3 µl Buffer D2 were added to a single cell on the cap of the collection tube, incubated for 10 min at 65 °C, and the reaction was terminated with 3 µl Stop solution. The reaction was supplemented with REPLI-g sc DNA polymerase and reaction buffer and incubated 30 °C for 8 hours. Amplified WGA DNA was stored at -20 °C. WGA amplicon quality was assessed by agarose gel electrophoresis and quantitative PCR (ViiA 7 system, Applied Biosystems) using Taqman primers 18S (Hs9999901_s1, Invitrogen) for Sanger sequencing and actin beta (ACTB) (Hs.PT.56a.40703009.g, Integrated DNA Technologies, Inc.) for next generation sequencing. WGA amplicons with Ct value above 25 in qPCR assay was not used for subsequent library construction and sequencing.

6.3.6 Sanger Sequencing

The protocol for Sanger sequencing was generously shared by the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre. I performed all the sample preparation including PCR and purification step, and the Sanger sequencing and analysis was performed by the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre. We used PCR to amplify the exon 8 locus of the androgen receptor (AR exon 8) from individual tumor cells using the following primers (Forward: 5'-CCA CCT CCT TGT CAA CCC TGT TTT TC-3', Reverse: 5'-ACA GAG ATG ATC TCT GCC ATC ATT TC-3'). The reaction mixture (final volume 25 µl) contained 1x Q5 Hot Start High-Fidelity Master Mix, 200 µM dNTP, 1 µM forward primers, 1 µM reverse primers, 0.5U Q5 Hot Start High-Fidelity DNA Polymerase and 25 ng of template DNA. Reactions were cycled: 98 °C-30 sec, 25X (98 °C-8 sec, 55 °C-10 sec, 72 °C-3 sec), 4 °C -forever. The PCR products were purified using the AMPure (Agencourt A63881) and sequenced using a Big Dye Terminator Cycle Sequencing kit and an ABI3100 DNA sequencer (Applied Biosystems, Foster City, CA). Chromas software was used to detect the T877A and H875Y mutations in LNCaP and 22RV1 cells, respectively.

6.3.7 Targeted Next Generation Sequencing

The targeted next generation sequencing analysis was performed by the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre. A custom NimbleGenSeqCap EZ Choice Library kit was used to generate a library for sequencing a panel of 73 genes relevant to prostate cancer (developed by Wyatt group at Vancouver Prostate Centre)¹⁹⁶. Following singlecell WGA, 10–100 ng of DNA was sheared into 180bp fragments with a Covaris focused ultrasonicator and modified by addition of 3' poly-A tail, end repair and adapter ligation. PCR

amplification (12-17 cycles) was performed and the resultant DNA was quantified by NanoDrop 2000 spectrophotometer and agarose electrophoresis. Libraries were purified with Agencourt AMPure beads and quantitated with the Qubit HS kit. Pools were diluted to 20 pM and sequenced on the Illumina MiSeq (v3 600 cycle kit). The low-quality bases from read tails and adapter were trimmed and reads were aligned against hg38 reference genome using Bowtie 2.2.4. Germline variants were called using samtools mpileup function, and the mutation was further filtered using the following criteria. A somatic mutation was called in a CTC sample if it had at least 5 supporting reads, and at least 20% of all reads at the site supported the mutation. The mutant allele fraction in the CTC sample was required to be at least 5 times higher than the allele fraction in the WBC sample, and the WBC sample was required to have at least 20 reads overlapping the site.

6.4 Development of Single-Cell Isolation and Analysis Workflow

6.4.1 **Fixation-Free Staining and DNA Quality Check**

In the previous section 5.7, CTC identification method using immunofluorescence techniques was developed. Here, the protocol is modified to stain cells using cell surface markers (e.g. EpCAM and CD45) in a fixation-free fashion in order to ensure cell integrity and the compatibility with downstream WGA. Conventionally, cells are fixed during the immunofluorescence process, resulting in potential DNA damage that reduces the efficiency of WGA¹⁹⁷. Initially, we investigated the potential DNA damage in UM-UC3-mCherry by



Figure 6.2 Potential DNA damage on PFA-fixed single cells after WGA. Fixation of specimens with PFA reduces the efficiency of subsequent WGA. The integrated transgene mCherry and endogenous 18S rDNA were targeted for quantification by qPCR. Target gene abundance was consistently lower in DNA isolates from PFA-treated cells, with mCherry loci only robustly detected in non-fixed UC3-mCherry specimens.

performing qPCR of the WGA products. Specifically, UM-UC3-mCherry cells were fixed with 4% paraformaldehyde for 10 min. Using the single-cell LCM process five individual fixed cells and five unfixed cells were isolated and subjected to WGA. Using qPCR, only fixation-free samples exhibited amplification of target genes (mCherry), while gene amplification from all PFA-treated cells failed (Figure 6.2), indicated by the higher Ct value. This suggests that PFA fixation introduces the potential DNA damage, which might interfere the amplification of DNA during WGA process.

Given the potential DNA damage using PFA-fixation, the new protocol was developed to perform the live-staining using cell surface markers, EpCAM for cancer cells and CD45 for leukocytes to distinguish CTCs from leukocytes. The detailed procedure for the fixation-free staining is available in Section 6.3.2. The live-staining process was tested by doping LNCaP prostate cultured cancer cells into whole blood. After scanning with a ZEISS LSM 780 imaging



LNCaPs: Hoechst+/EpCAM+/CD45- & Leukocytes: Hoechst+/EpCAM-/CD45+

Figure 6.3 Comparison of standard immunostaining of LNCaP tumor cells (left image) for CD45 (red), EpCAM (green) and Hoechst (blue), with live staining using the same antibodies. Live staining of tumor cells showed similar cell specificity and fluorescence intensity as tumor cells stained using the standard protocol.

system, both LNCaP and leukocytes were successfully identified as shown in figure 6.3.

Furthermore, live-stained samples showed no significant differences in staining efficiency when compared to the samples that are PFA-fixed and permeabilized except for slightly less efficiency of nuclear staining on leukocytes.

6.4.2 Cell Encapsulation Using a Hydrogel matrix

To extract single cells using laser capture microdissection (LCM), a cell encapsulation process using a hydrogel material was developed to create a 'tissue-like' substrate. Conventionally, LCM has been used to isolate and extract the tissue sections, which are typically dried and fixed on a glass slide. For cytological samples such as blood or cells in suspension, Cytospin is typically used to concentrate cells on the membrane¹⁹⁰. This approach is unsuitable for isolating rare cells, such as CTCs, because the centrifugation step introduces an unacceptable amount of loss to the cell sample¹⁹⁰. Furthermore, Cytospin-prepared slides are typically fixed with ethanol or paraformaldehyde, introducing potential DNA damage and further cell loss during the process¹⁹⁰. In our process, the cells are encapsulated in a 'tissue-like' hydrogel substrate, where they can be extracted using LCM. This process was initially tested using pre-stained UC13 cells doped into blood from healthy donors. After microfluidic enrichment, the cells were introduced to reservoirs in the PDMS baffle on a glass slide, followed by adding a hydrogel-forming polymer. The PDMS baffle allows the concentration and deposition of cells on a glass slide using centrifugation. The reservoirs in the PDMS baffle are designed to have a radius of 3 mm with 6mm in height, which is similar to the conventional 384-well plate for easier handling and scanning process. After centrifugation, the polymer was cured to form a hydrogel using a long wave UV (350 nm) for the minimum curing time for 25 seconds to prevent any DNA damage on our samples. After curing, cells in the PDMS baffle are distributed in a single layer and locked in the position during the entire scanning and extraction process for at least 4 hours. Thus, we created a 'tissue-like' substrate for samples in suspension that holds cells in a hydrogel layer to facilitate the cell extraction using LCM.

6.4.3 Single Cell Extraction using Laser Capture Microdissection

Individual cells were microdissected by laser pressure catapulting (LPC) using the PALM MicroBeam LCM System (Zeiss Microlaser Technologies, Munich, Germany). The extraction



Figure 6.4 Single cell extraction using LCM. A single UC-13 cell transfected with EGFP is marked and extracted using laser microdissection system. The captured cell is visually confirmed on a cap of a capture tube.

process was optimized using pre-stained UC13 cells embedded in PEGDA layer on a PEN membrane slide. The key factors for successful single-cell isolation using LCM are the concentration of cells in the reservoirs, and the thickness of PEGDA layer. First, we assessed the ideal concentration of cells embedded in a 3-mm reservoir of PDMS baffle. In order to isolate single cells using LCM, cells should not be too close each other to give sufficient space for laser cutting around a single cell. A range of 5,000 to 10,000 cells in a reservoir was experimentally determined to be ideal to facilitate the single-cell extraction using LCM. Second, the thickness of PEGDA layer was optimized by varying the amount of PEGDA mixture solution used to encapsulate cells, keeping the UV curing time for 25 seconds. Cells were encapsulated in a 3mm reservoir with 4, 5, and 6 μ l of the PEGDA solution and excised by the LCM system. By controlling the laser power between 35 to 45 % in order to avoid possible sample damage, we concluded that PEGDA layer created using 5 µl was the best to encapsulate and extract single cells in our process. Less than 5 µl of PEGDA solution created a thinner layer, resulting a loss of cells during scanning and extracting process. More than 5 µl of PEGDA solution provided the thickest layer, and the catapulting the target cells onto the collection tube was not efficient.

In order to verify the extraction process, UM-UC13-EFFP cells were embedded in a PEGDA layer created using 5 μ l of PEGDA solution on a PEN membrane slide. Samples were prepared on the PEN membrane slides, which can be easily cut together with the sample. PEN membrane also acts as a stabilizing support during lifting. Individual cells (n=30) were imaged both on the PEN membrane as well as on the AdhesiveCap of the collection tube in order to confirm the collection of the target cells (Figure 6.4). In all instances, the cell could be efficiently excised from the hydrogel and >90% of excised cells were captured on the collection cap.

6.4.4 **CTC Enumeration using a Customized Software**

In the previous section 5.7.2, multispectral analysis for CTC enumeration was discussed. While spectral analysis offers more sensitive and quantitative analysis, this process was performed manually by selecting the region-of-interest and check for the spectral data, which can be



Figure 6.5 Gigapixel Spectral Image Cube. Peak channels are listed as well as the quality control bright field channel which is obtained from the transmitted light through the sample while all color channels are fluorescent emissions from the sample.

[reproduced from Richard Ang's Thesis²¹¹]



Figure 6.6 Review software for CTC enumeration.. [reproduced from Richard Ang's Thesis²¹¹]

laborious and time-consuming. Thus, a customized spectral image cytometry software that allows a rapid CTC enumeration was developed by Richard Ang during his master thesis¹⁸¹. Specifically, this software analyzes the images obtained from the Zeiss LSM 780 system to interpret spectral data and to rank individual events based on the likelihood of being CTCs. When the samples are scanned using the Zeiss LSM 780 system, a gigapixel image cube of 32 different spectral channels is generated (Figure 6.5). Using the gigapixel spectral image cube and the pre-defined emission peak criteria, the software analyzes the spectral data and ranks cells based on their spectral emission from the most likely to least likely to be a CTC. The software then generates a simple interface for users to manually review the emission spectrum for each individual cell (Figure 6.6). After the reviewing process, a 'CTC map' is generated, which contains the positional information of each target cells for further single-cell extraction process using LCM. Since imaging ranking is automated, the process dramatically reduces the review time to identify and enumerate CTCs from each sample from hours to a few minutes, demonstrating its potential to use in clinical application.

6.5 Validation for Single-Cell Analysis Workflow

6.5.1 **Demonstration of Pure Tumor Cells Separations from a Mixed Population**

To establish that this process could precisely extract genetic material from a heterogeneous cell source, we generated 1:1 mixture of UM-UC13-EGFP and UM-UC3-mCherry cells and evaluated them for evidence of contaminant genetic material. A mixture of cells was embedded



Figure 6.7 (A) Single UC13 cell extraction using LCM. A single UC-13 cell transfected with EGFP is marked and extracted using laser microdissection system. The captured cell is visually confirmed on a cap of a capture tube. (B, C) Two cell lines UM-UC13-EGFP and UM-UC3-mCherry were intentionally combined in suspension and four single cells from each cell line, as well as a no-template (NT) and cell line reference DNA (gDNA) controls, were subjected to WGA and analyzed by transgene-specific qPCR. The black arrows indicate where qPCR failed to amplify the target DNA locus Successful extraction of target cells was indicated by detection of that cell's specific transgene but not the transgene of the contaminant cell line. in a PEGDA layer, and four single cells from each cell lines were extracted using LCM. The samples were individually inspected using fluorescence microscopy to confirm that only a single cell had been isolated as well as to verify its cell origin based on fluorescence. Identify of cells were confirmed using fluorescence microscopy (Figure 6.7A). Single cells were collected on the AdhesiveCap (Zeiss), followed by applying lysis buffer directly inside the cap. The collection tubes were kept in an inverted position during the lysis step, ensuring the DNA retrieval from a single cell for further amplification process using WGA. Using a pooled population as reference, DNA transgene abundance were assessed by qPCR assay. In all cases, single UM-UC13-EGFP and UM-UC3-mCherry cells harbored genetic material for only the transgene that corresponded with the fluorescence phenotype (Figure 6.7B and C). These data suggest that LCM excised individual cells and that non-target contaminant genetic material did not confound single cell WGA.

6.5.2 Validation of Single-cell Analysis Workflow using Spiked Sample

To evaluate the entire workflow, we combined RBC lysis, microfluidic enrichment, live-staining, hydrogel encapsulation, and LCM excision of single cells, into a single experimental workflow (Figure 6.8A). This workflow was optimized and validated using cultured tumor cells (e.g. 22RV1 and LNCaP) doped into whole blood. Following RBC lysis and microfluidic ratchet deformability-based enrichment, samples were live-stained and embedded in PEGDA hydrogel on a PEN Membrane slide. Prior to LCM extraction, the target cells were identified using the customized software. Using the interface, spectral data on each image was inspected in order to generate a 'CTC map' of target cells for extraction in <10 min (Figure 6.8B). The CTC map

contains the positional coordinates for target cells that were used to relocate these individual cells for isolation using LCM.

To verify that specific tumor cells could be isolated from a heterogeneous blood sample in a manner that enabled identification of their distinct genetic characteristics, doping experiments



Figure 6.8 (A) Single-cell analysis workflow. (B) Target cells are identified using our customized software, and the positional information was stored as a CTC map. The CTC map was used to identify target cells for single cell extraction using LCM. (C) Following WGA, we successfully amplified the AR exon 8 region by PCR from single LNCaP (L1-L3) and single 22RV1 (R1 and R2) cells, as well as CD45+ leukocytes within the enriched specimen (W1 and W2). An unoccupied segment of the PEGDA membrane was excised as a negative control

(P). (D) Sanger sequencing of the PCR products revealed the previously established T877A and H874Y mutations in LNCaP and 22RV1, respectively, while the leukocyte amplicons (WBC) displayed the wildtype sequence.

were performed using prostate cancer cell lines. LNCaP and 22RV1 are both prostate cancer cell lines with characteristic mutations in AR. LNCaP have a T877A mutation and 22RV1 have a H874Y mutation¹⁹⁸. Overall ~100 tumor cells were doped into the whole blood and processed the sample using the enrichment and isolation protocol. Three LNCaP and two 22RV1 cells were excised by LCM along with two contaminant leukocytes, to serve as controls. Following WGA, we performed PCR spanning the AR exon 8 and obtained an amplicon for both leukocytes and two of each tumor cells (Figure 6.8C). Sanger sequencing confirmed that the leukocyte cells had the wildtype AR sequence while both LNCaP and 22RV1 displayed their distinct defining mutations (Figure 6.8D). Additionally, cell-free hydrogel regions (indicated as P in the figure 6.8C) were excised and amplified using the same AR primer to establish that our experiment was not confounded by contaminant cell-free DNA.

6.5.3 Validation of Single-cell Analysis Workflow using Next Generation Sequencing

To further evaluate the compatibility of LCM-based single-cell isolation with next generation sequencing, we performed a targeted sequencing in both bulk cells and WGA DNA from single cells using a custom Roche Nimblegen EZ SeqCap panel and downstream Illumina sequencing machine. Single cells from two cancer cell lines, UM-UC13-EGFP and UM-UC3 mCherry, were



Figure 6.9 Scatter plots demonstrating the concordance between each pair of samples for mutations or single nucleotide polymorphism (SNP) present at >5% frequency in UC13 and UC3 bulk cell lines. [This figure was generated by Alexander Wyatt in the Wyatt Prostate Genomics Laboratory.]



Figure 6.10 Single cell analysis validation using targeted next generation sequencing.

isolated and DNA was amplified using WGA and sequenced. High single nucleotide polymorphism (SNP) concordance between a single cell sequence with both another single cell as well as with pooled specimens of the same clonal origin were observed while examining SNP that were present at >5% frequency in either cell line. (Figure 6.9). There was poor SNP concordance between UC13-GFP and UC3-mCherry, when comparing single cells to either individual cells of the alternate clone or to a pooled specimen (Figure 6.9). The SNPs that were detected included numerous cell line specific tumor mutations, including KRAS, RB1, and TP53 (Figure 6.10). Together, these results indicate that LCM-extracted cells are compatible with NGS genomic analysis and that this analysis can both identify tumor-defining SNPs and discriminate between tumor types.

6.6 Discussion and Summary

The growing interest in the sequencing of individual CTCs stems from a limitation for pooled CTC analysis as this approach may lead to false observations and interpretations due to contamination with non-CTC cells such as leukocytes²⁸. While single-cell sequencing can overcome these limitations, existing methods are subject to various technical challenges and these difficulties are compounded by the fact that CTCs are exceedingly rare. To address these challenges, an efficient workflow to isolate and characterize single cells that can be coupled with microfluidic separation was developed. After microfluidic enrichment, cells in suspension were live-stained and scanned using Zeiss LSM 780 imaging system. Scanned images were analyzed by our customized software to generate the 'CTC map' that contains the locations of target cells. Target cells were then extracted using PALM Microbeam LCM system, and WGA was performed. Using various downstream genotyping assays such as qPCR, Sanger sequencing and NGS, we confirmed that our single-cell analysis workflow is capable of isolating and characterizing single cells.

A key challenge in single-CTC sequencing is that these cells are extremely rare and may be lost or may lose genetic integrity during enrichment and extraction. Flow cytometry was initially used for single cell isolation¹⁹⁹ but is not appropriate for CTC characterization because it requires a large number of input target cells. LCM is well established but significant cell loss were reported during the sample preparation for cytological samples using the Cytospin due to the nature of cytofunnel design. Furthermore, samples are typically treated with chemical fixation to identify the target region. Several studies have reported that paraformaldehyde fixation reduces

the efficiency of subsequent WGA ^{191,200}. By performing fixation-free staining and stabilizing cells in a porous hydrogel, efficient cell encapsulation and single cell extraction were achieved, while reducing the potential DNA damage due to fixation. Existing LCM single-cell extraction methods have also been criticized for the potential to lose rare CTCs as they are projected to the collection cap¹⁹¹. We used the AdhesiveCap (Zeiss) to collect single cells using LCM, providing the instant immobilization of samples inside the cap. By visual inspection, we confirmed that 93% of cultured cancer cells were successfully captured on the AdhesiveCap, which is comparable to the previous report using NanoVelcro chip (LCM transfer success rate= 98.8%)²⁰¹. Furthermore, we directly applied lysis buffer inside the cap while maintaining the tube in an inverted position for DNA recovery, without centrifugation step to collect and lyse cells in a tube, increasing our target cell recovery rate.

In summary, we developed a workflow for the isolation and sequencing of single CTCs. By combining a label-free selective enrichment process and LCM excision of live un-fixed cells, this workflow provides significant advantages over conventional single cell isolation strategies. Since this method is compatible with downstream next generation sequencing, it provides a valuable tool for evaluating tumor status or response to therapy. Furthermore, this rapid process for CTC genotyping could provide key insight into the heterogeneity of CTCs and their important role in the metastatic process.

Chapter 7: CTC separation, identification and single CTC sequencing in prostate cancer

7.1 Introduction

CTC analysis in prostate cancer is particularly important because the site of metastasis is predominantly in the bone, making biopsy difficult and low yield⁶⁵. As obtaining tumor tissue of bone is highly invasive and often technically challenging, such procedures are currently not part of routine clinical care of patients and sequential biopsy is almost impossible²⁰². In contrast to tissue biopsies, CTCs can be isolated from blood to provide non-invasive, rapid, and longitudinal access to key tumor cells relevant to metastasis. Since CTCs share genetic characteristics with the inaccessible metastatic tissue, they could enable identification of defining mutations associated with patients with prostate cancer to select the optimal course of treatment and to evaluate response to therapy.

Previously, we developed a microfluidic device that separates CTCs based on cell deformability, as well as an accompanying analytical pipeline to identify CTCs using multispectral imaging system. Our initial study on CTC morphology demonstrated that there is a significant size overlap between CTCs from patients with prostate cancer and leukocytes, suggesting the potential to separate CTCs based on deformability. In doping experiments, the microfluidic ratchet device had an enrichment of ~ 10^4 -fold with ~90% capture rate, representing significantly improved selectivity over other label-free methods that separate CTCs based on size alone. The microfluidic ratchet enrichment was coupled with a single-cell multispectral imaging system that is a more objective method to identify CTCs. Furthermore, a single-cell analysis workflow was
developed to isolate single CTCs from enriched samples by a label-free microfluidic device. In this workflow, the enriched cells were live-stained, encapsulated in a hydrogel material (e.g. PEGDA), and extracted using laser capture microdissection (LCM). The target cells were identified using our customized spectral flow cytometry software, which generates the 'CTC map' that contains the positional information. Using the cultured cancer cells, this workflow was able to isolate single tumor cells and perform the molecular characterization such as qPCR and genome sequencing. In this chapter, we describe the application of our CTC separation and single CTC sequencing technology to analyze blood samples from patients with metastatic and localized prostate cancer.

Section 7.2 describes the overall approach for our studies. Section 7.3 presents CTC separation results from patient with castration-resistant prostate cancer (CPRC). Section 7.4 presents CTC separation results from patients with localized prostate cancer in. Finally, Section 7.6 presents single CTC genome sequencing results from patients with castrate resistant prostate cancer (CRPC).

7.2 Overall Approach

CTCs from prostate cancer patients were separated using the microfluidic ratchet device that sorts cells based on deformability through tapered constrictions under oscillating flow. Enriched samples were identified using the multispectral analysis. In metastatic CRPC patients, the samples were processed in parallel using the conventional CellSearch system and the CTC count was compared with CellSearch count in order to evaluate and validate our device performance. In localized prostate cancer, we investigated if the presence or number of CTC is correlated with other clinical and pathologic risk parameters, including PSA, Gleason score, and pT-stage (analyzed by Tilman Todenhöfer). As for single-cell analysis workflow, we sequenced single CTCs along with the cell-free DNA (prepared by Wyatt group) from the same CRPC patient in order to validate our single-cell analysis workflow. We also sequenced the genomic DNA from bulk white blood cells to determine if isolated single CTCs are indeed from the same patient and to use it as a germline control in sequencing data analysis.

7.3 CTC Separation from Patients with Castration-Resistant Prostate Cancer

7.3.1 Experimental Section

7.3.1.1 Patient Sample Preparation and Processing

Blood samples was collected from patients with castration-resistant prostate cancer (CRPC) (n=20) into a CellSave[®] tube (Veridex), for CellSearch[®] analysis, or into 6-ml EDTA tubes (Becton- Dickinson), for microfluidic ratchet analysis. The whole blood was processed within 2 hours for the microfluidic ratchet device, and within 96 hours for the CellSearch[®] assay.

Overall, 2 mL of whole blood were processed using the continuous-flow microfluidic ratchet device (version 2.0). Each microfluidic device is capable of processing whole blood at a throughput of 1 mL per hour. Typically, two devices were used in parallel in patient experiments. Prior to sample processing, the microfluidic devices were filled with buffer solution (0.2 % Pluronic F127, Invitrogen) in PBS for 15 min to prevent nonspecific adsorption of cells to the wall of the device. Whole blood samples were infused at the left-bottom corner (sample inlet), while buffer solutions are introduced through buffer and oscillation inlets. Pressure-driven flow originating from sample and buffer inlets was controlled using a commercial pressure controller

(Fluigent, France), while flow from oscillation inlets was controlled using a custom-made pressure control system and software.

After processing, the enriched cells from the CTC reservoir were collected in a 15 mL falcon tube, and then washed using PBS by centrifuging 400 xg for 5 min. The cells were then stained using the protocol developed in Section 5.7. Briefly, cells were fixed with 4% of paraformaldehyde (PFA) in PBS for 15 min and washed with PBS. Next, the cells were permeabilized with 0.025% of Tween 20 in PBS for 10 min, washed with PBS, and blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour. The antibody cocktail, consisting of antipan-cytokeratin conjugated with Alexa 488 (CellSignaling), anti-EpCAM conjugated with Alexa 594 (CellSignaling), and anti-CD45 conjugated with APC (Biolegend), were then added and incubated at 4 °C overnight. Finally, the cells were washed with PBS twice, counterstained for DAPI (Vector Laboratory), and transferred to a 384-well optically transparent well plate for microscopy to identify CTCs. Stained cells were scanned with the Zeiss LSM 780 microscopy system. The entire well was imaged under a 40x oil immersion objective over 15x15 frames using a motorized stage. Each captured image consists of a 34 color spectral image including a transmission-photomultiplier (T-PMT) channel (bright field), a low wavelength PMT channel (DAPI), and 32 channels captured using a GaAsP spectral detector (CK/EpCAM/CD45).

7.3.1.2 CTC Identification

CTCs from CRPC patient samples were identified using the protocol developed in Section 5.7. Briefly, the process to identify CTCs from the spectral image involved first scanning for cells that showed bright green fluorescence indicating the presence of CK, without ring-shaped fluorescence emitted by CD45 on the cell surface. Each candidate CTC was then carefully analyzed using a region-of-interest capture of the spectral data of pixels encapsulating each candidate cell. CTCs were specifically classified based on an emission maximum at 521 nm (CK conjugated with Alexa 488) with clear DAPI signal and intact membrane shape from bright field image. All CTCs fitting this characteristic were also checked for a distinct peak at 618 nm for EpCAM expression. Leukocytes were classified with a spectral maximum at 660 nm (CD45 conjugated with APC) with a ring shape and clear DAPI signal. Finally, CTC counts obtained from 2 mL of patient blood using the microfluidic ratchet device were scaled to 7.5 mL to compare with results obtained using the CellSearch[®] assay.

The size of CTCS and leukocytes from CRPC patients were measured from the bright field images of the separated cells in suspension by drawing a circle on the outer edge of each target cells. At least 25 CTCs and leukocytes were measured for each CRPC data set.

7.3.1.3 CellSearch Analysis

Blood samples for the CellSearch[®] system were collected in a CellSave tube (Veridex), and 7.5 mL of mCRPC patient blood samples were processed within 96 hours of collection. This system first enriched CTCs using anti-EpCAM magnetic beads, and labeled the enriched cells using DAPI, CK-phycoerythrin (PE), and CD45-APC. The enriched cells were deposited in the CellTracks magnet, and imaged using the CellTracks Analyzer. Finally, a certified technician in the Clinical Assay laboratory at Vancouver Prostate Centre visually reviewed the candidate cells to identify CTCs defined as DAPI+CK+CD45- cells.

7.3.2 Results and Discussion

Using the deformability-based microfluidic device and analytical pipeline, CTCs from CRPC patients (n=20) were processed along with 4 healthy control donor samples. Patient-derived CTCs were stained for DAPI, CK, and EpCAM, as well as the leukocyte marker CD45 (Figure 7.1A and B). Using the multispectral imaging system (Zeiss LSM 780), CTCs were identified on the basis of an intact nucleus, expression of cytokeratin (CK), and the absence of expression of the leukocyte marker CD45, where they have clearly distinct fluorescence maxima for each fluorophore (Figure7.1B). Leukocytes were defined as DAPI+CD45+, where one peak is present at 660 nm for CD45+.

Based on these criteria, CTCs were enumerated from the immunofluorescence images. The CTC detection using the microfluidic ratchet device was significantly higher with a median 178 CTCs/7.5 mL, compared to a median 7 CTCs/7.5 mL with CellSearch[®] (Figure 7.1C and D, p=0.0031). Interestingly, the number of CTCs detected after microfluidic enrichment did not correlate with the number of detected by CellSearch[®]. This discordance likely derives from the fact the biophysical separation is likely to access a different population of CTCs than immunomagnetic enrichment used by CellSearch[®].

To investigate the potential for false positives, blood samples from four healthy donors were processed, where a median of 4 false-positive cells/7.5 mL was detected. The existence of false positives is a common feature of all CTC identification methods, including the CellSearch[®] system. These cells potentially arise from epithelial cells sloughed into blood from venipuncture or tissue trauma, or from the expression of cytokeratins in leukocytes leukocytes²⁰³. While the



Figure 7.1 (A,B) Fluorescence microscopy image and corresponding spectra for CTCs (DAPI+CK+CD45-) and leukocytes (DAPI+CK-CD45+). (C,D) CTC enumeration comparison between the microfluidic ratchet device and the CellSearch[®] system. The p-value indicates a significant difference by Wilcoxon-ranked sum. (E) Comparison of cell size between leukocytes and patient-derived CTCs, which showed no statistically difference.

presence of false positives limits the ultimate specificity of the system, the number of false 121

positives was significantly lower than the median CTC number in patient samples.

Label-free enrichment of CTCs from patients with prostate cancer represented a significant challenge because CTCs from patients with prostate cancer tend to have cell sizes that overlap significantly with leukocytes^{103,106,162}. Based on our model of the relationship between size only and combination of size and deformability sorting in doped tumor cells, we hypothesized that sensitive enrichment of patient derived depended on the contribution of deformability in the separation of these cells. To evaluate this, the diameter of enriched CTCs as well as the diameter of patient leukocytes before and after enrichment was measured. Consistent with our hypothesis, the contaminating leukocytes in the collection outlet were not significantly larger than the pre-enriched population. Furthermore, there was significant overlap in size between CTCs and leukocytes, suggesting that these cells were primarily discriminated on the basis of their cell deformability (Figure 7.1E). This permitted greater sensitivity in detecting CTCs in patients with CRPC than CellSearch[®].

In addition to the highly selective enrichment of CTCs, we employed a system that combined sensitive enrichment by microfluidic ratchet with single celled multi-spectrum imaging. Multispectrum imaging improves upon standard CTC identification by limiting false positives that occurs due to spectral overlap, autofluorescence signal and provides the opportunity for quantitative and automated scoring of suspected CTCs. When combined with the sensitive microfluidic ratchet enrichment mechanism, this CTC enumeration system identified 25-fold more CTCs than conventional CellSearch[®] analysis.

Another contributing factor to the sensitivity of CTC capture by microfluidic ratchet is that it is not limited on the basis of EpCAM expression. Although all CTC from CRPC patients in our experiment were EpCAM positive, expression level of EpCAM varies from different patients and cells. Previous studies also suggested that CTCs from the same patients exhibit heterogeneous expression of EpCAM⁹⁹. Furthermore, loss or downregulation of epithelial markers (e.g. EpCAM) are often associated with CTCs that may undergo the epithelial-to-mesenchymal transition^{25–27}. EpCAM-based immunoaffinity capture of CTCs requires high cell surface expression the antigen and these conventional methods may fail to capture CTCs with low levels of EpCAM expression²². Because of the high sensitivity of the confocal microscope and spectral analysis may have contributed to detect CTCs with low expression of EpCAM. In fact, CTC counts obtained using deformability based separation show a high discordance with CellSearch CTC counts. This discordance may be originated from potential low EpCAM expressing CTCs that are enriched by microfluidic ratchet, which may have been missed by CellSearch[®] due to the threshold for efficient immunocapture.

Overall, we demonstrated that our deformability-based microfluidic ratchet device is able to capture >25-fold more CTCs than the established CellSearch[®] system, while the separated cells remain in suspension and are available for downstream characterization.

7.4 CTC Separation from Patients with Localized Prostate Cancer

7.4.1 Experimental Section

7.4.1.1 Patient Sample Preparation and Processing

Overall 50 samples from patients with undergoing radical prostatectomy for clinically localized prostate cancer were collected into a 5-ml EDTA tube and processed within 24 hours of collection. For each patient, 2 ml of whole blood was processed using the continuous-flow microfluidic device in the same way described in Section 7.3.

7.4.1.2 CTC Enrichments from Patients with Localized Prostate Cancer

After microfluidic enrichment, cells were stained with CK-Alexa488, EpCAM-Alexa594 and CD45-APC, and counter-stained with DAPI in the same way described in Section 7.3. A subset of localized prostate cancer patient samples (n=21) were stained also with an anti-androgen receptor (AR) antibody conjugated with Alexa 555 (CellSignaling). Stained cells were transferred to a 384-well optically transparent plate, and scanned with the Zeiss LSM 780 microscopy system. CTCs were identified by 2 reviewers based on their emission spectra as CK+/CD45- whereas leukocytes were identified as CD45+. EpCAM positivity was not used as requirement to determine whether a cell was a CTC.

7.4.1.3 Statistical Analysis

Results from 2 different reviewers were correlated by linear regression analysis. The mean count of the 2 reviewers were correlated to pathological and clinical variables by the Wilcoxon-Mann-Whitney test for continuous parameters and the Chi-square test for categorical parameters. JMP 7.0 (SAS Inc. Cary, NC) was used. P<0.05 was considered significant.



Figure 7.2 Immunofluorescence images and spectral analysis for CTCs (DAPI+CK+CD45-) and leukocytes(DAPI+CK-CD45-) from localized prostate cancer patients.

7.4.2 **Results and Discussion**

Blood samples from a total of 50 localized prostate cancer patients were processed and analyzed. CTCs were identified using the spectral image analysis by 2 independent reviewers The CTC counts by two reviewers were not significantly different (p<0.0001), and the average of two counts were reported. Although we did not use EpCAM expression to define CTC, all CTCs identified were EpCAM positive. In small subset patients (n=20), cells were additionally stained for androgen receptor (AR) (Figure 7.2).

Clinical and histopathological parameters and risk groups are summarized in Table 7.1. From a total of 50 patients, 25 (50%) had at least 1 CTC present in 2 mL of whole blood. Overall, 12 patients had \geq 5 CTC in 2 mL of whole blood and 2 patients had >50 CTC. The median CTC count in CTC-positive patients was 4.5 CTC/mL (range 0.5-208.5). Although we identified CTCs in 50% of patient with localized prostate cancer using our label-free microfluidic ratchet

device, the presence of CTC did not correlate with pathologic other clinical and pathologic risk parameters, including PSA concentration, pathologic tumor stage (pT), lymph node stage (N), Gleason score, and or risk category. Similarly, in patients with positive CTC counts, the number of CTC was not associated with pT-stage, N-stage, or Gleason score (Table 7.2).

Table 7.1 Patient characteristics in whole cohort, CTC positive and CTC negative patients.

	All patients (n=50)	CTC negative patients (n=25)	CTC positive patients (n=25)	p-value
Age, Median (Range)	66 (51-76)	65 (53-76)	67 (51-75)	0.36
PSA preoperative,	7.4 ng/ml (1.2-25)	7.4 (1.2-25.0)	7.5 (1.4-17.0)	0.89
Median (Range)				
Gleason Biopsy				0.72
6	6 (12%)	2 (8%)	4 (16%)	
7a	22 (44%)	12 (48%)	10 (40%)	
7b	13 (26%)	5 (20%)	8 (32%)	
8	6 (12 %)	4 (16%)	2 (8%)	
9	3 (6 %)	2 (8%)	1 (4%)	
Risk group				0.52
Low Risk	6 (12 %)	2 (8%)	4 (16%)	
Intermediate Risk	35 (70 %)	17 (68%)	18 (72%)	
High Risk	9 (18 %)	6 (24%)	3 (12%)	
Gleason final patholo				0.36
gу				
6	4 (8%)	1 (4%)	3 (12%)	
7a	28 (56%)	15 (60%)	13 (52%)	
7b	14 (28%)	6 (36%)	8 (32%)	
8	1 (2%)	0 (0%)	1 (4%)	
9	2 (4%)	2 (8%)	0 (0%)	
10	1 (2%)	1 (4%)	0 (0%)	
pT-stage				0.70
2a	4 (8%)	1 (4%)	3 (12%)	
2b	2 (4%)	1 (4%)	1 (4%)	
2c	31 (62%)	16 (84%)	15 (60%)	
3a	9 (18%)	4 (16%)	5 (20%)	
3b	4 (8%)	3 (12%)	1 (4%)	
N-stage				0.54
NO	47 (94%)	23 (92%)	24 (96%)	
N1	3 (6%)	2 (8%)	1 (4%)	

[data analysis performed by Tilman Todenhöfer.]

Table 7.2 Correlation of CTC counts with pathologic risk parameters.

	Median CTC count / ml (Range)	p-value
pT stage		
pT < 3	0 (0-57)	0.84
pT ≥ 3	0.5 (0-208.5)	
pN stage		
pN0	0.5 (0-208.5)	0.17
pN+	0 (0-3)	
Gleason Score		
Gleason ≤ 7	0.5 (0-208.5)	0.51
Gleason = 7	0 (0-0.5)	

[data analysis performed by Tilman Todenhöfer.]

In metastatic CRPC, CTC count is a strong predictor of overall survival and has predictive value. For example, Danila et al. reported that a higher baseline CTC count was significantly associated with worse survival⁴⁹. Moreover, a change in CTC count after treatment was associated with survival⁵¹. Unlike metastatic CRPC, the role of CTC analysis in localized prostate cancer has not been fully established. Although we were able to detect CTCs in 50% of patients with localized prostate cancer using a label-free microfluidic device, the correlation with other clinical and pathological risk parameters was not clear. This observation is consistent with other previous studies^{68,69,75}. For example, in a study of 97 of patients with localized prostate cancer, Davis et al. detected CTCs in 21% of localized prostate cancer patient samples using CellSearch^{®69}. Similar to our result, they did not observe any correlation with established clinical and pathologic risk parameters. Likewise, Thalgott et al. identified only 1 CTC/7.5 mL in 1 out of 20 patients (5%) with localized prostate cancer using CellSearch^{®68}. In a subsequent study from the same group reported that 3/15 (20%) patients with localized prostate cancer undergoing neoadjuvant docetaxel chemotherapy had at least one CTC in 20 mL of whole blood²⁰⁴. Again, there was no correlation between CTC counts and clinicopathologic risk parameters. Furthermore, Meyer et al. identified CTCs in 11 % of patients before radical prostatectomy (total number of sample = 152) using CellSearch[®] assay⁷⁵. Similarly, authors could not find the significant correlation of presence of CTC with T-stage, Gleason score or PSA level.

Although our microfluidic ratchet device had a higher CTC counts compared to other studies using CellSearch[®], we were not able to determine if those CTCs from our device are in fact malignant cells derived from the primary prostate cancer. While our microfluidic ratchet device isolates blood cells independent of cell surface antigen, we have used immunofluorescence to

define the CTC. We have employed the most common definition of CTC, which is a peripheral blood cell with a round or oval morphology, an intact nucleus and expression of CK but not CD45. Several studies suggest that a portion of CK+CD45- cells indeed possess the characteristics of cancer cells^{205,206}. However, all markers for detection of CTC in prostate cancer, including CK and PSA, have provided false positive results in healthy donors^{23,69}, indicating that at least a subset of these CK+CD45- cells does not represent cancer cells. Since our microfluidic device depends only on the biophysical properties of the cells, if alternative surface biomarkers are identified the system could be easily adapted to include them in analysis to improve the specificity to CTC detection.

In summary, a high number of CTCs can be detected in a considerable portion of patients with clinically localized prostate cancer using our microfluidic ratchet device. This indicates that techniques based on EpCAM enrichment might miss a significant proportion of CTC. Similar to many other studies in localized prostate cancer, CTC counts observed in our study did not correlate with clinical and pathologic risk parameters.

7.5 Single-Cell Sequencing of CTCs from Patients with Castration-Resistance Prostate Cancer

7.5.1 Experimental Section

7.5.1.1 Patient Blood Sample Collection

Blood was collected from patients with metastatic castration resistant prostate cancer (n=11) with written consent and approval by the BC Cancer Agency ethics committee. Patient blood (8 ml)

was collected using EDTA tubes (BD, Franklin Lakes, NJ) and was stored at room temperature for processing within 24 hours.

7.5.1.2 Red Blood Cell Lysis

Blood samples were collected in EDTA tubes, and red blood cells were lysed using RBC lysis buffer (G-bioscience) according to the manufacturer's protocol. Briefly, the lysis buffer was added to the blood samples at 1: 3 ratios (v/v). The sample was incubated for 5 min on the rotating rack and centrifuged down at 2500 xg for 5 min. After the centrifugation, the supernatant was carefully removed without disturbing the pellet. The cell pellets were resuspended in 1/15th initial blood volume with 3% BSA in PBS solution.

7.5.1.3 Microfluidic Ratchet-Based Cell Sorting

The multiplexed inlet microfluidic devices were made using a standard technique of photolithograph and soft lithography as previously described in Section 5.4. Prior to operation, the microfluidic ratchet device was pre-incubated with phosphate buffered saline (PBS) with 0.2% Pluronic F127 (Invitrogen) to prevent non-specific adsorption of cells to microstructures of the device. Cell samples were infused through the sample inlet, while buffer solutions were infused through the buffer and oscillation inlets. Pressure-driven flow originating from these inlets was controlled using a custom-made pressure control system and software, described previously.

7.5.1.4 CTC Identification and Encapsulation using a Hydrogel matrix

Tumor cells were identified by fixation-free live-cell staining, described previously in Section 6.3. Briefly, the enriched CTCs were washed once with PBS and incubated with 3% BSA in PBS for 30 min at room temperature without fixation. Antibody cocktail including anti-EpCAM antibody conjugated with Alexa 488 (CellSignaling), anti-CD45 antibody conjugated with APC (Biolegend), and Hoechst 33342 (Invitrogen) was added to cells and incubated for 2 hours at room temperature. Subsequently, cells were washed with PBS, and placed in the wells of PDMS baffle on a PEN membrane glass slide, followed by overlaying the PEGDA solutions (5 μ l). The sample was centrifuged (2740 x g, 3 min) and cured under UV (wavelength = 375 nm) for 25 sec. Unpolymerized PEGDA was removed by pipette and replaced with 15 μ l DNase-free water to prevent desiccation. Embedded samples were then scanned using the Zeiss LSM 780 microscopy system. CTC candidates were identified by our customized software system.

7.5.1.5 Single CTC Extraction and Sequencing

Single CTCs were extracted as described previously in Section 6.4. Briefly, single CTC candidates were extracted using the PALM MicroBeam laser capture microdissection system (Zeiss, Thornwood, NY), and WGA was performed to amplify DNA. The WGA DNA was subjected to DNA quality check with qPCR targeting for actin beta (ACTB). For qualified WGA DNA, we employed a targeted sequencing strategy using a custom NimbleGenSeqCap EZ Choice Library and Illumina MiSeq (v3 600 cycle kit) machine as previously described in Section 6.4. The sequencing work was performed by the Wyatt Prostate Genomics Laboratory at the Vancouver Prostate Centre. The white blood cell samples were prepared using the DNeasy

Blood & Tissue Kits (Qiagen) from enriched cells collected on leukocytes outlet from our microfluidic device.

As for the sequencing data analysis, the low-quality bases from read tails and adapter were trimmed and reads were aligned against hg38 reference genome using Bowtie 2.2.4. Germline variants were called using the samtools mpileup function, and the mutation was further filtered using the following criteria. A somatic mutation was called in a CTC sample if it had at least 5 supporting reads, and at least 20% of all reads at the site supported the mutation. The mutant allele fraction in the CTC sample was required to be at least 5 times higher than the allele fraction in white blood cells sample, and the white blood cells sample was required to have at least 20 reads overlapping the site.

7.5.2 **Results and Discussion**

Using the multiplexed inlet microfluidic device (version 2.1) and CTC enumeration software, CTCs were detected in 3 out of 11 patients with an average count of 87 CTCs per 8 mL of blood. Whole blood was collected into an 8 ml EDTA tube and subjected to red blood cell lysis before tumor cell enrichment by microfluidic ratchet sorting and live-immunostaining for EpCAM and CD45, and counter stained for Hoechst. The live-stained sample was encapsulated in PEGDA hydrogel. CTCs were enumerated using the customized software, and defined as EpCAM+CD45- cells with an intact nucleus (Figure 7.3A and B). In order to validate the single cell sequencing workflow, a patient sample with CTC counts (VC023) as well as cell-free DNA sample was selected for further single-cell isolation and analysis. In this sample, a total of 191 predicted CTCs were identified and eight of these cells were isolated by LCM for further



Figure 7.3 (A) CTCs (DAPI+/EpCAM+/CD45-), enriched from blood of patients with castration resistant prostate cancer, were imaged alongside contaminant leukocytes (WBCs; DAPI+/EpCAM-/CD45+). (B) Spectral analysis discriminated CTCs based on the CTC-specific peak at 520 nm (EpCAM-Alexa488) and the WBCspecific peak 660 nm (CD45-APC). (C) Image illustrating the extraction of a single CTC using the LCM system, as well as the confirmed collection of this cell on a cap of the collection tube. (D) Following single cell extraction and WGA, a qPCR was performed to amplify the ACTB locus as a confirmation of template quality. Five CTCs qualified for subsequent next generation sequencing.

characterization. Each excised CTC was successfully collected on the collection cap (Figure

7.3C) and was subjected to whole genome amplification (WGA). After qPCR verification of WGA amplification product (Figure 7.3D), five cells were selected for NGS analysis.

Before NGS library construction we measured WGA efficiency by qPCR assay as the conventional gel electrophoresis could not determine if the DNA is properly amplified in our case. According to REPLI-g WGA product protocol, the negative control will also be amplified as Phi29 DNA Polymerase has an extremely high processivity and will extend primer-dimers that may be present in the reaction, leading to non-specific amplification products. In fact, we observed a smear on a negative control, containing a piece of PEGDA layer extracted by LCM. However, the amplification from a PEGDA layer did not interfere with our downstream genetic analysis, as no signal was detected in the qPCR assay as well as in the Sanger sequencing as previously described in Section 6.5. For the CPRC patient (VC023), we attempt to collect 9 CTCs, and 8 of 9 CTCs were successfully collected on the AdhesiveCap. Among collected CTCs, 5 out of 8 (62.5 %) single cells passed DNA quality test using qPCR targeting for actin beta (ACTB).

NGS analysis employed a custom Roche Nimblegen EZ SeqCap panel across 72 cancer-related genes and downstream Illumina sequencing. To restrict our analysis to somatic mutations, single nucleotide polymorphism (SNP) that were not present in paired leukocytes were evaluated. Across these five CTCs, 45 somatic mutations were identified within this panel, spanning 24 genes. Most (35) of the somatic mutations were restricted to individual cells, while nine mutations were identified in two cells (PTEN, FOXA1, ATR, KMT2C, FANCC) and a single TP53 mutation was present in 4 of 5 isolated CTCs (Figure 7.4). As a reference, we



Figure 7.4 Next generation sequencing was performed to compare cell free DNA (cfDNA) with single CTCs and a single leukocyte (WBC). A) Forty five somatic mutations were detected that were not present in the reference WBC. B) Eleven mutations were shared by more than 2 single CTCs. In comparison with cfDNA, only three genes (TP53, PTEN, and FOXA1) exhibited identical mutations. C) TP53 mutation was detected in 4 out of 5 single CTCs and, when present, was found in virtually all reads, as illustrated as a fraction of mutant sequences over total reads. D) PTEN somatic mutation was detected in 2 out of 5 single CTC samples, however the low number of reads available for this gene suggests that this locus may not have been wellrepresented among the WGA amplicons.

simultaneously sequenced cell free DNA from paired patient blood samples using the same panel library. The cfDNA sequence showed concordance with 11 mutations identified for the single CTCs. These included the TP53, PTEN and FOXA1 mutations (Figure 7.4). Together, these analyses indicated that while some mutations can be identified from a cfDNA pool, single CTCs may accumulate relevant mutations that are not reflected in the cfDNA sequence.

One possible reason for the discrepancy in sensitivity for detection of mutations, between cfDNA and single CTCs, may reflect the fact that cfDNA derives from a genetically heterogeneous population. We observed that the TP53 and PTEN mutations were only present in 55% and 60% of cfDNA reads, respectively (Figure 7.4C and D). Conversely, in CTCs where TP53 mutations were detected they reflected >88% of reads. In CTC #1, PTEN mutation was detected in 44% of reads but was represented in >99% of reads from CTC #3. Consequently, it is conceivable that relevant mutations may missed in cfDNA specimens because they are obscured by non-target contaminant genetic material.

The key goals of clinical CTC genotyping are to use CTCs as a surrogate for the originating tumor when establishing an accurate prognosis or evaluating the response of the tumor to therapy. The value of this strategy is supported by >50% concordance between mutations of primary or metastatic tumors and the isolated CTCs in both prostate³³ and colon cancer ³⁰. While tissue biopsy is typically a difficult and invasive procedure, the isolation of CTCs from peripheral blood represents a compelling non-invasive alternative that can be performed repeatedly to monitor tumor response to therapy. Consistent with this goal, we developed a workflow to enrich for CTCs from whole patient blood using highly sensitive microfluidic device, live-immunostain the sample, and isolate single CTCs using laser capture microdissection system. To validate the potential application of this single cell extraction

process, we performed next generation sequencing of five single CTCs from a patient with metastatic prostate cancer. Paired sequencing of single CTCs alongside cfDNA demonstrated that, as reported in previous reports ²⁰⁷, individual cells share defining somatic mutations (e.g. TP53, PTEN, FOXA1) common to the circulating tumor population. However, the CTCs also had mutations not detected in cfDNA. These mutations may be relevant because they may reflect relevant CTC subpopulations that differ in their metastatic potential or response to therapy. Consequently, a more extensive analysis of patient CTCs could provide important insight into the metastatic cascade.

7.6 Summary

In this chapter, CTCs were separated using our label-free microfluidic device based on cell deformability. In patients with metastatic CRPC, where CTCs are not significantly larger than leukocytes, CTCs can be captured based on deformability at 25x greater yield than with the conventional CellSearch[®] system. In patient with localized prostate cancer, we detected CTCs in 50% of patients with significantly higher CTC counts compared to reported studies. Furthermore, single CTCs from a CRPC patient were isolated using laser capture microdissection and sequenced using the targeted next generation sequencing, revealing the key mutations (e.g. PTEN, TP53) in prostate cancer, and the heterogeneity of CTCs.

Chapter 8: Conclusions and Future Work

8.1 Summary of Results

This dissertation describes the development of a technology to enrich for CTCs, an analytical pipeline to identify CTCs, a workflow to extract single CTCs for genome sequencing, as well as the application of these technologies and processes to study patients with metastatic castrationresistant prostate cancer (CRPC) and localized prostate cancer. Initially, a study on the morphology of CTCs from patients with prostate cancer was performed to observe that CTCs and leukocytes were similar in size, but distinct in nucleus-to-cytoplasm ratio. These results suggested the potential to separate CTCs based on deformability. Based on this result, a microfluidic device that separates CTCs based on cell deformability, as well as an accompanying analytical pipeline to identify CTCs using immunofluorescence, were developed, optimized, and tested. This workflow was used to successfully enumerate CTCs from 20 patients with metastatic castrate resistant prostate cancer, as well as 25 patients with localized prostate cancer. For the former cohort, we compared our process against established technology and demonstrated $25 \times$ greater yield for CTCs. Finally, we developed a single CTC isolation process using laser capture microdissection for genome sequencing. Using this process, 5 single CTCs from a patient with prostate cancer were sequenced using the targeted next generation sequencing. The sequencing data confirmed the presence of major driver mutations, including PTEN and TP53, as well as heterogeneous characteristics of individual CTCs. Together, this workflow demonstrated its potential to isolate, extract, and sequence single CTCs from patients with prostate cancer in order to discover clinically relevant mutations that may be useful in monitoring disease progression and guiding patient treatment.

8.2 Future Work

Having developed a robust workflow sequencing of single CTCs, a key goal toward applying this technology in a clinical setting would involve refinement of this process to enable analysis of more patient samples. Specifically, this process can be improved by automating CTC enumeration and isolation steps through the use of software or robotics. The current technologies are limited by the need to obtain the scanned images from Zeiss LSM 780 confocal microscopy and analyze them using our customized CTC enumeration software. Although this software significantly reduced a processing time for CTC identification using automated spectral analysis, each CTC candidate has to be marked and relocated manually for CTC isolation using PALM MicroBeam laser microdissection system. Ideally, we could modify our CTC enumeration software in laser microscopy. This step would enable the automatic relocation of the target cells and extraction of multiple cells in a multi-well plate instead of using single collection tubes for higher throughput. As an efficient process becomes available, single CTCs could be collected in excess of what is needed to biobank CTCs samples for future characterization.

In addition to enhancing the efficiency of the workflow, the process can be modified to adopt transcription profiling of CTCs instead of genomic profiling. Transcriptome profiling may prove more challenging than DNA sequencing because RNA is a less stable molecule. Furthermore, single cell RNA analysis typically involves with extra PCR steps to generate cDNA and purification process compared to a simple one-step DNA amplification process for a single-cell genomic analysis. Fortunately, there have been recent technological advances in single cell RNA sequencing methods that address this issue. Notably, the single-cell tagged reverse transcription

(STRT) and template-switching (e.g. SMART RNA-seq)^{208–210} have reportedly enabled fulllength transcriptome coverage from single cells, identifying single-nucleotide polymorphism (SNP)²¹⁰. By utilizing this available RNA-seq method, combined with our existing CTC separation and single cell analysis technology may be able to profile transcriptome of single prostate cancer CTCs. Some key insights that could be gained from transcription profiling include the ability to monitor changes in gene expression, following therapy, or to investigate important RNA biomarkers, such as the alternatively spliced transcripts ARv7 and microRNA expression.

With improved single-cell analysis workflow as well as transcriptome profiling established, further clinical studies could be performed in a larger scale to identify potential genetic markers for disease progression and drug response. Initially, more patient samples with castrationresistant prostate cancer could be obtained and processed using our improved single-cell analysis workflow to extract single CTCs and perform transcriptome profiling. The sequencing analysis can be performed in parallel with cfDNA and primary and metastatic tumor analysis (as available) for more complete genomic data. This genomic data can be analyzed to compare copy number and sequence variation in various stages of cancer progression in order to correlate CTC genotype with patients' outcome, to monitor the response to specific drug, and to guide therapeutic choices. The value of this study could also extend to localized prostate cancer for early disease detection and upfront treatment. Ultimately, the comparative genomic data from key single CTCs, tumor tissues, and cfDNA will provide distinct perspective from which to identify new biomarkers for disease status and treatment efficacy.

Bibliography

- Crnic, I. & Christofori, G. Novel technologies and recent advances in metastasis research. *International Journal of Developmental Biology* 48, 573–581 (2004). DOI: 10.1387/ijdb.041809ic
- Gupta, G. P. & Massagué, J. Cancer Metastasis: Building a Framework. *Cell* 127, 679– 695 (2006). DOI: 10.1016/j.cell.2006.11.001
- Attard, G. *et al.* Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. *Cancer Res.* 69, 2912–2918 (2009). DOI: 10.1158/0008-5472.CAN-08-3667
- Beltran, H. *et al.* Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. *Eur. Urol.* 63, 920–926 (2013). DOI: 10.1016/j.eururo.2012.08.053
- Eccles, S. A. & Welch, D. R. Metastasis: recent discoveries and novel treatment strategies. Lancet 369, 1742–1757 (2007). DOI: S0140-6736(07)60781-8 [pii]\r10.1016/S0140-6736(07)60781-8 [doi]
- de Bono, J. S. *et al.* Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin. Cancer Res.* 14, 6302–9 (2008). DOI: 10.1158/1078-0432.CCR-08-0872
- Cristofanilli, M. *et al.* Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 351, 781–791 (2004). DOI: 10.1056/NEJMoa040766\r351/8/781 [pii]

- Budd, G. T. *et al.* Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. *Clin. Cancer Res.* 12, 6403–6409 (2006). DOI: 10.1158/1078-0432.CCR-05-1769
- Cohen, S. J. *et al.* Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J. Clin. Oncol.* 26, 3213–3221 (2008). DOI: 10.1200/JCO.2007.15.8923
- Lim, E. *et al.* Clinical results of microfluidic antibody-independent peripheral blood circulating tumor cell capture for the diagnosis of lung cancer. *J. Thorac. Cardiovasc. Surg.* 147, 1936–1938 (2014). DOI: 10.1016/j.jtcvs.2013.09.052
- Qi, F. *et al.* Quantitation of rare circulating tumor cells by folate receptor α ligand-targeted
 PCR in bladder transitional cell carcinoma and its potential diagnostic significance. *Tumor Biol.* 35, 7217–7223 (2014). DOI: 10.1007/s13277-014-1894-0
- 12. Jin, T. *et al.* Clinical value of circulating liver cancer cells for the diagnosis of hepatocellular carcinoma: A meta-analysis. *Biomed. Reports* 1, 731–736 (2013). DOI: 10.3892/br.2013.139
- Okegawa, T. *et al.* Immunomagnetic quantification of circulating tumor cells in patients with urothelial cancer. *Int. J. Urol.* 17, 254–8 (2010). DOI: 10.1111/j.1442-2042.2010.02454.x
- 14. Carden, C. P. *et al.* Can molecular biomarker-based patient selection in Phase I trials accelerate anticancer drug development? *Drug Discovery Today* 15, 88–97 (2010). DOI: 10.1016/j.drudis.2009.11.006

- Reid, A. H. M. *et al.* Significant and sustained antitumor activity in post-docetaxel, castration-resistant prostate cancer with the CYP17 inhibitor abiraterone acetate. *J. Clin. Oncol.* 28, 1489–1495 (2010). DOI: 10.1200/JCO.2009.24.6819
- Maheswaran, S. & Haber, D. A. Circulating tumor cells: a window into cancer biology and metastasis. *Current Opinion in Genetics and Development* 20, 96–99 (2010). DOI: 10.1016/j.gde.2009.12.002
- Salvianti, F. *et al.* Single circulating tumor cell sequencing as an advanced tool in cancer management. *Expert Rev. Mol. Diagn.* 7159, 14737159.2016.1116942 (2015). DOI: 10.1586/14737159.2016.1116942
- Riethdorf, S. *et al.* Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin. Cancer Res.* 13, 920–928 (2007). DOI: 10.1158/1078-0432.CCR-06-1695
- 19. Miller, M. C. *et al.* Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. *J. Oncol.* 2010, 1–8 (2010). DOI: 10.1155/2010/617421
- Shaffer, D. R. *et al.* Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. *Clin. Cancer Res.* 13, 2023–9 (2007). DOI: 10.1158/1078-0432.CCR-06-2701
- Nagrath, S. *et al.* Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 450, 1235–1239 (2007). DOI: 10.1038/nature06385
- Ozkumur, E. *et al.* Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. *Sci. Transl. Med.* 5, 179ra47 (2013). DOI: 10.1126/scitranslmed.3005616

- Stott, S. L. *et al.* Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc. Natl. Acad. Sci.* 107, 18392–18397 (2010). DOI: 10.1073/pnas.1012539107
- Allard, W. J. *et al.* Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.* 10, 6897–6904 (2004). DOI: 10.1158/1078-0432.CCR-04-0378
- Paterlini-Brechot, P. & Benali, N. L. Circulating tumor cells (CTC) detection: clinical impact and future directions. *Cancer Lett.* 253, 180–204 (2007). DOI: 10.1016/j.canlet.2006.12.014
- Willipinski-Stapelfeldt, B. *et al.* Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. *Clin. cancer Res. an Off. J. Am. Assoc. Cancer Res.* 11, 8006–8014 (2005). DOI: 10.1158/1078-0432.CCR-05-0632
- Zhang, L. *et al.* The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci. Transl. Med.* 5, 180ra48 (2013). DOI: 10.1126/scitranslmed.3005109
- Sieuwerts, A. M. *et al.* Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast Cancer Res. Treat.* 118, 455–468 (2009). DOI: 10.1007/s10549-008-0290-0 [doi]
- Leung, M. L. *et al.* Highly multiplexed targeted DNA sequencing from single nuclei. *Nat. Protoc.* 11, 214–235 (2016). DOI: 10.1038/nprot.2016.005

- Heitzer, E. *et al.* Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. *Cancer Res.* 73, 2965–2975 (2013). DOI: 10.1158/0008-5472.CAN-12-4140
- Ni, X. *et al.* Reproducible copy number variation patterns among single circulating tumor cells of lung cancer patients. *Proc. Natl. Acad. Sci. U. S. A.* 110, 21083–8 (2013). DOI: 10.1073/pnas.1320659110
- Dago, A. E. *et al.* Rapid phenotypic and genomic change in response to therapeutic pressure in prostate cancer inferred by high content analysis of single Circulating Tumor Cells. *PLoS One* 9, (2014). DOI: 10.1371/journal.pone.0101777
- 33. Lohr, J. G. *et al.* Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nat. Biotechnol.* 32, 479–484 (2014). DOI: 10.1038/nbt.2892
- 34. Lohr, J. G. *et al.* Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nat. Biotechnol.* 32, 479–484 (2014). DOI: 10.1038/nbt.2892
- Langley, R. R. & Fidler, I. J. The seed and soil hypothesis revisited-The role of tumorstroma interactions in metastasis to different organs. *Int. J. Cancer* 128, 2527–2535 (2011). DOI: 10.1002/ijc.26031
- Fidler, I. J. Timeline: The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat. Rev. Cancer* 3, 453–458 (2003). DOI: 10.1038/nrc1098
- Joosse, S. A. *et al.* Biology, detection, and clinical implications of circulating tumor cells.
 EMBO Mol. Med. 7, 1–11 (2015). DOI: 10.15252/emmm.201303698

- 38. Thiery, J. P. Epithelial–mesenchymal transitions in tumour progression. *Nat. Rev. Cancer*2, 442–454 (2002). DOI: 10.1038/nrc822
- Thiery, J. P. & Lim, C. T. Tumor dissemination: An EMT affair. *Cancer Cell* 23, 272–273 (2013). DOI: 10.1016/j.ccr.2013.03.004
- 40. Kalluri, R. EMT: When epithelial cells decide to become mesenchymal-like cells. *Journal of Clinical Investigation* **119**, 1417–1419 (2009). DOI: 10.1172/JCI39675
- 41. Kallergi, G. *et al.* Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. *Breast Cancer Res.* 13, R59 (2011). DOI: 10.1186/bcr2896
- 42. Yu, M. *et al.* Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science (80-.).* 339, 580–584 (2013). DOI: 10.1126/science.1228522
- 43. Krebs, M. G. *et al.* Molecular analysis of circulating tumour cells—biology and biomarkers. *Nat. Rev. Clin. Oncol.* 11, 129–144 (2014). DOI: 10.1038/nrclinonc.2013.253
- Thiery, J. P. *et al.* M. A. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* 139, 871–890 (2009). DOI: 10.1016/j.cell.2009.11.007
- 45. Ashworth, T. R. A case of cancer in which cells similar to those in the tumors were seen in the blood after death. *Aust. Med. J.* **14**, 146–149 (1869). DOI:
- 46. Cristofanilli, M. *et al.* Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J. Clin. Oncol.* 23, 1420–30 (2005). DOI: 10.1200/JCO.2005.08.140

- 47. Hayes, D. F. *et al.* Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin. Cancer Res.* 12, 4218–4224 (2006). DOI: 10.1158/1078-0432.CCR-05-2821
- Cohen, S. J. *et al.* Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients With Metastatic Colorectal Cancer. *J. Clin. Oncol.* 26, 3213–3221 (2008). DOI: 10.1200/JCO.2007.15.8923
- Danila, D. C. *et al.* Circulating tumor cell number and prognosis in progressive castrationresistant prostate cancer. *Clin. Cancer Res.* 13, 7053–7058 (2007). DOI: 10.1158/1078-0432.CCR-07-1506
- 50. de Bono, J. S. *et al.* Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin. Cancer Res.* 14, 6302–6309 (2008).
 DOI: 10.1158/1078-0432.CCR-08-0872 [doi]
- de Bono, J. S. *et al.* Abiraterone and increased survival in metastatic prostate cancer. *N. Engl. J. Med.* 364, 1995–2005 (2011). DOI: 10.1056/NEJMoa1014618
- Cohen, S. J. *et al.* Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients With Metastatic Colorectal Cancer. *J. Clin. Oncol.* 26, 3213–3221 (2008). DOI: 10.1200/JCO.2007.15.8923
- Danila, D. C. *et al.* Phase II multicenter study of abiraterone acetate plus prednisone therapy in patients with docetaxel-treated castration-resistant prostate cancer. *J. Clin. Oncol.* 28, 1496–501 (2010). DOI: 10.1200/JCO.2009.25.9259
- 54. Scher, H. I. *et al.* Circulating tumor cell biomarker panel as an individual-level surrogate for survival in metastatic castration-resistant prostate cancer. *J. Clin. Oncol.* 33, 1348–1355 (2015). DOI: 10.1200/JCO.2014.55.3487

- 55. Gerlinger, M. *et al.* Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N. Engl. J. Med.* 366, 883–892 (2012). DOI: 10.1056/NEJMoa1113205
- Jensen, J. D. *et al.* PIK3CA mutations may be discordant between primary and corresponding metastatic disease in breast cancer. *Clin. Cancer Res.* 17, 667–677 (2011). DOI: 10.1158/1078-0432.CCR-10-1133
- 57. Nieto, M. A. et al.. EMT: 2016. Cell 166, 21–45 (2016). DOI: 10.1016/j.cell.2016.06.028
- 58. Wu, S. *et al.* Classification of circulating tumor cells by epithelial-mesenchymal transition markers. *PLoS One* **10**, (2015). DOI: 10.1371/journal.pone.0123976
- 59. Quin, F. W. & Mead, A. J. Application of single-cell genomics in cancer: Promise and challenges. *Human Molecular Genetics* **24**, R74–R84 (2015). DOI: 10.1093/hmg/ddv235
- Qian, W. *et al.* Capturing Cancer: Emerging Microfluidic Technologies for the Capture and Characterization of Circulating Tumor Cells. *Small* 11, 3850–3872 (2015). DOI: 10.1002/smll.201403658
- Hotte, S. J. & Saad, F. Current management of castrate-resistant prostate cancer. *Curr. Oncol.* 17, (2010). DOI: 10.3747/co.v17i0.718
- Ferlay, J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 127, 2893–2917 (2010). DOI: 10.1002/ijc.25516
- 63. Harris, W. P. *et al.* Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. *Nat. Clin. Pract. Urol.* 6, 76–85 (2009). DOI: 10.1038/ncpuro1296

- An Advanced Prostate Cancer Diagnosis ... Now What ? (2015). Available at:
 http://www.prostatecancer.ca/Prostate-Cancer/About-Prostate-Cancer/Advanced-Prostate-Cancer. DOI:
- 65. León-Mateos, L., *et al.* Clinical application of circulating tumour cells in prostate cancer: From bench to bedside and back. *International Journal of Molecular Sciences* 17, (2016). DOI: 10.3390/ijms17091580
- 66. Tombal, B. & Lecouvet, F. Modern detection of prostate cancer's bone metastasis: Is the bone scan era over? *Advances in Urology* (2012). doi:10.1155/2012/893193 DOI: 10.1155/2012/893193
- 67. Chen, J.-F. *et al.* Circulating tumor cells in prostate cancer: beyond enumeration. *Clin. Adv. Hematol. Oncol.* 15, 63–73 (2017). DOI: 10.3390/cancers5041676
- Thalgott, M. *et al.* Detection of circulating tumor cells in different stages of prostate cancer. *J. Cancer Res. Clin. Oncol.* 139, 755–763 (2013). DOI: 10.1007/s00432-013-1377-5
- 69. Davis, J. W. *et al.* Circulating tumor cells in peripheral blood samples from patients with increased serum prostate specific antigen: initial results in early prostate cancer. *J. Urol.* 179, 2187–91; discussion 2191 (2008). DOI: 10.1016/j.juro.2008.01.102
- Stott, S. L. *et al.* Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer. *Sci. Transl. Med.* 2, 25ra23 (2010). DOI: 10.1126/scitranslmed.3000403
- 71. Leversha, M. A. *et al.* Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. *Clin. Cancer Res.* 15, 2091–2097 (2009). DOI: 10.1158/1078-0432.CCR-08-2036

- Miyamoto, D. T. *et al.* Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. *Cancer Discov.* 2, 995–1003 (2012). DOI: 10.1158/2159-8290.CD-12-0222
- Jiang, R. *et al.* A comparison of isolated circulating tumor cells and tissue biopsies using whole-genome sequencing in prostate cancer. *Oncotarget* 6, 44781–44793 (2015). DOI: 10.18632/oncotarget.6330
- 74. Miyamoto, D. T. *et al.* RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. *Science (80-.).* 349, 1351–1356 (2015). DOI: 10.1126/science.aab0917
- Meyer, C. P. *et al.* Limited prognostic value of preoperative circulating tumor cells for early biochemical recurrence in patients with localized prostate cancer. *Urol. Oncol.* 34, 235.e11-235.e16 (2016). DOI: 10.1016/j.urolonc.2015.12.003
- Pal, S. K. *et al.* Detection and phenotyping of circulating tumor cells in high-risk localized prostate cancer. *Clin. Genitourin. Cancer* 13, 130–136 (2015). DOI: 10.1016/j.clgc.2014.08.014
- Went, P. T. *et al.* Frequent EpCam Protein Expression in Human Carcinomas. *Hum. Pathol.* 35, 122–128 (2004). DOI: 10.1016/j.humpath.2003.08.026
- Pantel, K. *et al.* Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat. Rev. Cancer* 8, 329–340 (2008). DOI: 10.1038/nrc2375
- 79. Talasaz, A. H. *et al.* Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. *Proc. Natl. Acad. Sci.* 106, 3970–3975 (2009). DOI: 10.1073/pnas.0813188106

- Cann, G. M. *et al.* mRNA-Seq of Single Prostate Cancer Circulating Tumor Cells Reveals Recapitulation of Gene Expression and Pathways Found in Prostate Cancer. *PLoS One* 7, (2012). DOI: 10.1371/journal.pone.0049144
- Kirby, B. J. *et al.* Functional characterization of circulating tumor cells with a prostatecancer-specific microfluidic device. *PLoS One* 7, (2012). DOI: 10.1371/journal.pone.0035976
- 82. Galletti, G. *et al.* Isolation of breast cancer and gastric cancer circulating tumor cells by use of an anti HER2-based microfluidic device. *Lab Chip* 14, 147–56 (2013). DOI: 10.1039/c3lc51039e
- 83. Thege, F. I. *et al.* Microfluidic immunocapture of circulating pancreatic cells using parallel EpCAM and MUC1 capture: characterization, optimization and downstream analysis. *Lab Chip* 14, 1775–84 (2014). DOI: 10.1039/c4lc00041b
- 84. Sheng, W. *et al.* Capture, release and culture of circulating tumor cells from pancreatic cancer patients using an enhanced mixing chip. *Lab Chip* 14, 89–98 (2014). DOI: 10.1039/c3lc51017d
- 85. Harb, W. *et al.* Mutational Analysis of Circulating Tumor Cells Using a Novel Microfluidic Collection Device and qPCR Assay. *Transl. Oncol.* 6, 528–IN1 (2013). DOI: 10.1593/tlo.13367
- 86. Winer-Jones, J. P. *et al.* Circulating tumor cells: Clinically relevant molecular access based on a novel CTC flow cell. *PLoS One* 9, (2014). DOI: 10.1371/journal.pone.0086717
- Aktas, B. *et al.* Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res.* 11, R46 (2009). DOI: 10.1186/bcr2333
- Armstrong, A. J. *et al.* Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. *Mol. Cancer Res.* 9, 997– 1007 (2011). DOI: 10.1158/1541-7786.MCR-10-0490
- Joosse, S. a *et al.* Changes in keratin expression during metastatic progression of breast cancer: impact on the detection of circulating tumor cells. *Clin. Cancer Res.* 18, 993–1003 (2012). DOI: 10.1158/1078-0432.CCR-11-2100
- 90. Raimondi, C. *et al.* Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Res Treat* 130, 449–455 (2011).
 DOI: 10.1007/s10549-011-1373-x
- Pecot, C. V. *et al.* A novel platform for detection of CK + and CK CTCs. *Cancer Discov.* 1, 580–586 (2011). DOI: 10.1158/2159-8290.CD-11-0215
- 92. Mikolajczyk, S. D. *et al.* Detection of EpCAM-negative and cytokeratin-negative circulating tumor cells in peripheral blood. *J. Oncol.* (2011). doi:10.1155/2011/252361
 DOI: 10.1155/2011/252361
- 93. Marrinucci, D. *et al.* Fluid biopsy in patients with metastatic prostate, pancreatic and breast cancers. *Phys. Biol.* **9**, 16003 (2012). DOI: 10.1088/1478-3975/9/1/016003
- 94. Serrano, M. J. *et al.* EMT and EGFR in CTCs cytokeratin negative non-metastatic breast cancer. *Oncotarget* **5**, 7486–97 (2014). DOI: 10.18632/oncotarget.2217
- 95. Andreopoulou, E. *et al.* Comparison of assay methods for detection of circulating tumor cells in metastatic breast cancer: AdnaGen AdnaTest BreastCancer Select/DetectTM versus

Veridex CellSearchTM system. *Int. J. Cancer* **130**, 1590–1597 (2012). DOI: 10.1002/ijc.26111

- Liu, Z. *et al.* Negative enrichment by immunomagnetic nanobeads for unbiased characterization of circulating tumor cells from peripheral blood of cancer patients. *J. Transl. Med.* 9, 70 (2011). DOI: 10.1186/1479-5876-9-70
- 97. Fusi, A. *et al.* Monitoring of circulating tumor cells in a patient with synchronous metastatic melanoma and colon carcinoma. *Annals of Oncology* 21, 1734–1735 (2010).
 DOI: 10.1093/annonc/mdq330
- Karabacak, N. M. *et al.* Microfluidic, marker-free isolation of circulating tumor cells from blood samples. *Nat. Protoc.* 9, 694–710 (2014). DOI: 10.1038/nprot.2014.044
- 99. Ozkumur, E. *et al.* Inertial Focusing for Tumor Antigen-Dependent and -Independent Sorting of Rare Circulating Tumor Cells. *Sci. Transl. Med.* 5, 179ra47-179ra47 (2013).
 DOI: 10.1126/scitranslmed.3005616
- Shapiro, H. M. *et al.* Combined blood cell counting and classification with fluorochrome stains and flow instrumentation. *J. Histochem. Cytochem.* 24, 396–401 (1976). DOI: 10.1177/24.1.56391
- 101. Phillips, K. G. *et al.* Quantification of cellular volume and sub-cellular density fluctuations: comparison of normal peripheral blood cells and circulating tumor cells identified in a breast cancer patient. *Front. Oncol.* 2, (2012). DOI: 10.3389/fonc.2012.00096
- Ruban, G. I. *et al.* Investigation of morphometric parameters for granulocytes and lymphocytes as applied to a solution of direct and inverse light-scattering problems. *J. Biomed. Opt.* 12, 44017 (2007). DOI: 10.1117/1.2753466

- Coumans, F. A. W. *et al.* Filter Characteristics Influencing Circulating Tumor Cell
 Enrichment from Whole Blood. *PLoS One* 8, (2013). DOI: 10.1371/journal.pone.0061770
- Harouaka, R. A. *et al.* Circulating Tumor Cell Enrichment Based on Physical Properties. *J. Lab. Autom.* 18, 455–468 (2013). DOI: 10.1177/2211068213494391
- 105. Vona, G. *et al.* Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells. *Am. J. Pathol.* 156, 57–63 (2000). DOI: 10.1016/S0002-9440(10)64706-2
- 106. Park, S. *et al.* Morphological differences between circulating tumor cells from prostate cancer patients and cultured prostate cancer cells. *PLoS One* 9, (2014). DOI: 10.1371/journal.pone.0085264
- 107. Dong, C. *et al.* Cytoplasmic rheology of passive neutrophils. *Biorheology* 28, 557–567 (1991). DOI:
- 108. Ruef, P. *et al.* Deformability and volume of neonatal and adult leukocytes. *Pediatr. Res.*29, 128–132 (1991). DOI: 10.1203/00006450-199102000-00004
- Guo, Q. *et al.* Microfluidic micropipette aspiration for measuring the deformability of single cells. *Lab Chip* 12, 2687–95 (2012). DOI: 10.1039/c2lc40205j
- Meng, S. *et al.* Circulating tumor cells in patients with breast cancer dormancy. *Clin. Cancer Res.* 10, 8152–8162 (2004). DOI: 10.1158/1078-0432.CCR-04-1110
- 111. Chinen, L. T. D. *et al.* Cytokeratin-based CTC counting unrelated to clinical follow up. *J. Thorac. Dis.* 5, 593–599 (2013). DOI: 10.3978/j.issn.2072-1439.2013.09.18
- 112. Farace, F. *et al.* A direct comparison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. *Br. J. Cancer* 105, 847–853 (2011).
 DOI: 10.1038/bjc.2011.294

- Khoja, L. *et al.* A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. *Br. J. Cancer* 106, 508–516 (2012). DOI: 10.1038/bjc.2011.545
- Morris, K. L. *et al.* Circulating biomarkers in hepatocellular carcinoma. *Cancer Chemother. Pharmacol.* 74, 323–332 (2014). DOI: 10.1007/s00280-014-2508-7
- 115. Desitter, I. *et al.* A new device for rapid isolation by size and characterization of rare circulating tumor cells. *Anticancer Res.* **31**, 427–441 (2011). DOI: 31/2/427 [pii]
- 116. Freidin, M. B. *et al.* An assessment of diagnostic performance of a filter-based antibody-independent peripheral blood circulating tumour cell capture paired with cytomorphologic criteria for the diagnosis of cancer. *Lung Cancer* 85, 182–185 (2014). DOI: 10.1016/j.lungcan.2014.05.017
- Mu, Z. *et al.* Detection and Characterization of Circulating Tumor Associated Cells in Metastatic Breast Cancer. *Int. J. Mol. Sci.* 17, (2016). DOI: 10.3390/ijms17101665
- 118. Zheng, S. *et al.* 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. *Biomed. Microdevices* 13, 203–213 (2011). DOI: 10.1007/s10544-010-9485-3
- 119. Zhou, M.-D. *et al.* Separable Bilayer Microfiltration Device for Viable Label-free Enrichment of Circulating Tumour Cells. *Sci. Rep.* 4, 7392 (2015). DOI: 10.1038/srep07392
- Lin, H. K. *et al.* Portable filter-based microdevice for detection and characterization of circulating tumor cells. *Clin. Cancer Res.* 16, 5011–5018 (2010). DOI: 10.1158/1078-0432.CCR-10-1105

- 121. Chudziak, J. *et al.* Clinical evaluation of a novel microfluidic device for epitopeindependent enrichment of circulating tumour cells in patients with small cell lung cancer. *Analyst* 141, 669–678 (2016). DOI: 10.1039/C5AN02156A
- 122. Xu, L. *et al.* Optimization and evaluation of a novel size based circulating tumor cell isolation system. *PLoS One* **10**, (2015). DOI: 10.1371/journal.pone.0138032
- 123. Guo, Q. *et al.* Deterministic microfluidic ratchet based on the deformation of individual cells. *Phys. Rev. E Stat. Nonlinear, Soft Matter Phys.* 83, (2011). DOI: 10.1103/PhysRevE.83.051910
- 124. Park, E. S. *et al.* Continuous Flow Deformability-Based Separation of Circulating Tumor Cells Using Microfluidic Ratchets. *Small* 12, 1909–1919 (2016). DOI: 10.1002/smll.201503639
- Huang, L. R. Continuous Particle Separation Through Deterministic Lateral Displacement. *Science (80-.).* 304, 987–990 (2004). DOI: 10.1126/science.1094567
- 126. Davis, J. A. *et al.* Deterministic hydrodynamics: Taking blood apart. *Proc. Natl. Acad. Sci.* 103, 14779–14784 (2006). DOI: 10.1073/pnas.0605967103
- 127. Loutherback, K. *et al.* Deterministic separation of cancer cells from blood at 10 mL/min.*AIP Adv.* 2, (2012). DOI: 10.1063/1.4758131
- 128. Di Carlo, D. Inertial microfluidics. Lab Chip 9, 3038 (2009). DOI: 10.1039/b912547g
- 129. Hur, S. C. *et al.* High-throughput size-based rare cell enrichment using microscale vortices. *Biomicrofluidics* **5**, (2011). DOI: 10.1063/1.3576780
- Sollier, E. *et al.* Size-selective collection of circulating tumor cells using Vortex technology. *Lab Chip* 14, 63–77 (2014). DOI: 10.1039/c3lc50689d

- 131. Dean, W. R. XVI. Note on the motion of fluid in a curved pipe. *London, Edinburgh, Dublin Philos. Mag. J. Sci.* 4, 208–223 (1927). DOI: 10.1080/14786440708564324
- Hou, H. W. *et al.* Isolation and retrieval of circulating tumor cells using centrifugal forces.
 Sci. Rep. 3, 1259 (2013). DOI: 10.1038/srep01259
- 133. Khoo, B. L. *et al.* Clinical validation of an ultra high-throughput spiral microfluidics for the detection and enrichment of viable circulating tumor cells. *PLoS One* 9, (2014). DOI: 10.1371/journal.pone.0099409
- Gascoyne, P. R. C. & Shim, S. Isolation of circulating tumor cells by dielectrophoresis.
 Cancers (Basel). 6, 545–579 (2014). DOI: 10.3390/cancers6010545
- Pohl, H. A. *et al.* Dielectrophoretic force: A comparison of theory and experiment. *J. Biol. Phys.* 6, 133–160 (1978). DOI: 10.1007/BF02328936
- Wang, X. B. *et al.* Cell separation by dielectrophoretic field-flow-fractionation. *Anal. Chem.* 72, 832–839 (2000). DOI: 10.1021/ac9909220
- 137. Gupta, V. *et al.* ApoStream???, a new dielectrophoretic device for antibody independent isolation and recovery of viable cancer cells from blood. *Biomicrofluidics* 6, (2012). DOI: 10.1063/1.4731647
- Balasubramanian, P. *et al.* Antibody-independent capture of circulating tumor cells of non-epithelial origin with the ApoStream® system. *PLoS One* 12, (2017). DOI: 10.1371/journal.pone.0175414
- 139. O'Shannessy, D. J. *et al.* Isolation of circulating tumor cells from multiple epithelial cancers with ApoStream® for detecting (or monitoring) the expression of folate receptor alpha. *Biomark. Insights* **11**, 7–18 (2016). DOI: 10.4137/BMIMI.S35075

- 140. Medoro, G. *et al.* CMOS-only sensors and manipulators for microorganisms. *Tech. Dig. -Int. Electron Devices Meet.* 415–418 (2000). doi:10.1109/IEDM.2000.904344 DOI: 10.1109/IEDM.2000.904344
- 141. Manaresi, N. *et al.* A CMOS Chip for Individual Cell Manipulation and Detection. in *IEEE Journal of Solid-State Circuits* 38, 2297–2305 (2003). DOI: 10.1109/JSSC.2003.819171
- 142. Pestrin, M. *et al.* Heterogeneity of PIK3CA mutational status at the single cell level in circulating tumor cells from metastatic breast cancer patients. *Mol. Oncol.* 9, 749–757 (2015). DOI: 10.1016/j.molonc.2014.12.001
- Gross, A. *et al.* Technologies for single-cell isolation. *International Journal of Molecular Sciences* 16, 16897–16919 (2015). DOI: 10.3390/ijms160816897
- 144. Dalerba, P. *et al.* Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nat. Biotechnol.* 29, 1120–1127 (2011). DOI: 10.1038/nbt.2038
- 145. Shapiro, E. *et al.* Single-cell sequencing-based technologies will revolutionize wholeorganism science. *Nat. Rev. Genet.* **14**, 618–630 (2013). DOI: 10.1038/nrg3542
- 146. Hu, P. *et al.* Single Cell Isolation and Analysis. *Front. cell Dev. Biol.* 4, 116 (2016). DOI: 10.3389/fcell.2016.00116
- 147. Swennenhuis, J. F. *et al.* Efficiency of whole genome amplification of single circulating tumor cells enriched by CellSearch and sorted by FACS. *Genome Med.* 5, 106 (2013).
 DOI: 10.1186/gm510
- 148. Neves, R. P. L. *et al.* Genomic High-Resolution Profiling of Single CKpos/CD45neg Flow-Sorting Purified Circulating Tumor Cells from Patients with Metastatic Breast

Cancer. *Clin. Chem.* (2014). doi:10.1373/clinchem.2014.222331 DOI: 10.1373/clinchem.2014.222331

- 149. Vandewoestyne, M. *et al.* Laser capture microdissection: Should an ultraviolet or infrared laser be used? *Anal. Biochem.* 439, 88–98 (2013). DOI: 10.1016/j.ab.2013.04.023
- 150. Vona, G. *et al.* Impact of Cytomorphological Detection of Circulating Tumor Cells in Patients with Liver Cancer. *Hepatology* **39**, 792–797 (2004). DOI: 10.1002/hep.20091
- 151. Pinzani, P. *et al.* Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecular analysis by laser microdissection. *Hum. Pathol.* 37, 711–718 (2006). DOI: 10.1016/j.humpath.2006.01.026
- 152. Lu, Y. T. *et al.* NanoVelcro chip for CTC enumeration in prostate cancer patients. *Methods* 64, 144–152 (2013). DOI: 10.1016/j.ymeth.2013.06.019
- 153. Zhao, L. *et al.* High-purity prostate circulating tumor cell isolation by a polymer nanofiber-embedded microchip for whole exome sequencing. *Adv. Mater.* 25, 2897–2902 (2013). DOI: 10.1002/adma.201205237
- 154. Dean, F. B. *et al.* Rapid amplification of plasmid and phage DNA using Phi29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Res.* 11, 1095–1099 (2001). DOI: 10.1101/gr.180501
- 155. Zhang, K. *et al.* Sequencing genomes from single cells by polymerase cloning. *Nat.Biotechnol.* 24, 680–686 (2006). DOI: 10.1038/nbt1214
- 156. Kalisky, T. *et al.* Genomic Analysis at the Single-Cell Level. *Annu. Rev. Genet.* 45, 431–445 (2011). DOI: 10.1146/annurev-genet-102209-163607

- 157. Peters, B. A. *et al.* Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. *Nature* **487**, 190–195 (2012). DOI: 10.1038/nature11236
- 158. De Luca, F. *et al.* Mutational analysis of single circulating tumor cells by next generation sequencing in metastatic breast cancer. *Oncotarget* 7, 1–11 (2016). DOI: 10.18632/oncotarget.8431
- 159. Fehm, T. *et al.* Methods for isolating circulating epithelial cells and criteria for their classification as carcinoma cells. *Cytotherapy* 7, 171–185 (2005). DOI: 10.1080/14653240510027082
- 160. Lazar, D. C. *et al.* Cytometric comparisons between circulating tumor cells from prostate cancer patients and the prostate-tumor-derived LNCaP cell line. *Phys. Biol.* 9, 16002 (2012). DOI: 10.1088/1478-3975/9/1/016002
- 161. K.N., C. *et al.* A randomized phase II study of OGX-427 plus prednisone versus prednisone alone in patients with chemotherapy-naive metastatic castration-resistant prostate cancer. *Journal of Clinical Oncology* **30**, no pagination (2012). DOI:
- 162. Ligthart, S. T. *et al.* Circulating Tumor Cells Count and Morphological Features in Breast,
 Colorectal and Prostate Cancer. *PLoS One* 8, e67148 (2013). DOI:
 10.1371/journal.pone.0067148
- 163. Tan, S. J. *et al.* Versatile label free biochip for the detection of circulating tumor cells from peripheral blood in cancer patients. *Biosens. Bioelectron.* 26, 1701–1705 (2010).
 DOI: 10.1016/j.bios.2010.07.054
- McFaul, S. M. *et al.* Cell separation based on size and deformability using microfluidic funnel ratchets. *Lab Chip* 12, 2369 (2012). DOI: 10.1039/c2lc21045b

- 165. Marrinucci, D. *et al.* Case study of the morphologic variation of circulating tumor cells. *Hum. Pathol.* 38, 514–519 (2007). DOI: 10.1016/j.humpath.2006.08.027
- Marrinucci, D. *et al.* Cytomorphology of circulating colorectal tumor cells:a small case series. *J. Oncol.* 2010, 861341 (2010). DOI: 10.1155/2010/861341
- 167. Kraeft, S. K. *et al.* Detection and analysis of cancer cells in blood and bone marrow using a rare event imaging system. *Clin. Cancer Res.* **6**, 434–42 (2000). DOI:
- Borgen, E. *et al.* Standardization of the immunocytochemical detection of cancer cells in BM and blood: I. establishment of objective criteria for the evaluation of immunostained cells. *Cytotherapy* 1, 377–388 (1999). DOI: 10.1080/0032472031000141283
- 169. Pierga, J.-Y. *et al.* Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. *Clin. Cancer Res.* **10**, 1392–1400 (2004). DOI:
- 170. Song, H. *et al.* Monte Carlo simulation of LNCaP human prostate cancer cell aggregation in liquid-overlay culture. *Biotechnol Prog* 19, 1742–1749 (2003). DOI: 10.1021/bp034061n [doi]
- 171. Shi, Y. *et al.* Rheological properties of mammalian cell culture suspensions: Hybridoma and HeLa cell lines. *Biotechnol. Bioeng.* **41**, 745–54 (1993). DOI: 10.1002/bit.260410709
- 172. Breier, A. *et al.* Time dependence of [3H]-vincristine accumulation by L1210 mouse leukemic cells. Effect of P-glycoprotein overexpression. *Gen. Physiol. Biophys.* 13, 287–298 (1994). DOI:
- 173. Méhes, G. *et al.* Circulating Breast Cancer Cells Are Frequently Apoptotic. *Am. J. Pathol.*159, 17–20 (2001). DOI: 10.1016/S0002-9440(10)61667-7

- Matrone, M. A. *et al.*. Microtentacles tip the balance of cytoskeletal forces in circulating tumor cells. *Cancer Research* 70, 7737–7741 (2010). DOI: 10.1158/0008-5472.CAN-10-1569
- 175. Weiss, L. Biomechanical interactions of cancer cells with the microvasculature during hematogenous metastasis. *Cancer Metastasis Rev.* 11, 227–235 (1992). DOI: 10.1007/BF01307179
- 176. Cristofanilli, M. *et al.* Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 351, 781–791 (2004). DOI: 10.1056/NEJMoa040766\r351/8/781 [pii]
- Guilak, F. *et al.* Viscoelastic Properties of the Cell Nucleus. *Biochem. Biophys. Res. Commun.* 269, 781–786 (2000). DOI: 10.1006/bbrc.2000.2360
- Seal, S. H. Silicone flotation: A simple quantitative method for the isolation of free-floating cancer cells from the blood. *Cancer* 12, 590–595 (1959). DOI: 10.1002/1097-0142(195905/06)12:3<590::AID-CNCR2820120318>3.0.CO;2-N
- 179. Zheng, S. *et al.* Membrane microfilter device for selective capture, electrolysis and genomic analysis of human circulating tumor cells. *J. Chromatogr. A* 1162, 154–161 (2007). DOI: 10.1016/j.chroma.2007.05.064
- 180. Vona, G. *et al.* Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells. *Am. J. Pathol.* 156, 57–63 (2000). DOI: 10.1016/S0002-9440(10)64706-2
- 181. Yan, J. P. Development of an epoxy-based microfluidic device for automated circulating tumour cell separation. (2017). doi:10.14288/1.0354561 DOI: 10.14288/1.0354561

- 182. Gorges, T. M. *et al.* Heterogeneous PSMA expression on circulating tumor cells: a potential basis for stratification and monitoring of PSMA-directed therapies in prostate cancer. *Oncotarget* 7, 34930–34941 (2016). DOI: 10.18632/oncotarget.9004
- 183. Crnalic, S. *et al.* Nuclear androgen receptor staining in bone metastases is related to a poor outcome in prostate cancer patients. *Endocr. Relat. Cancer* 17, 885–895 (2010). DOI: 10.1677/ERC-10-0059
- 184. Altin, J. G. & Sloan, E. K. The role of CD45 and CD45-associated molecules in T cell activation. *Immunol. Cell Biol.* **75**, 430–445 (1997). DOI: 10.1038/icb.1997.68
- 185. Hofman, V. J. *et al.* Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method: promises and pitfalls. *Am. J. Clin. Pathol.* 135, 146–156 (2011). DOI: 10.1309/AJCP9X8OZBEIQVVI
- 186. Saliba, A.-E. *et al.* Single-cell RNA-seq: advances and future challenges. *Nucleic Acids Res.* 42, 8845–60 (2014). DOI: 10.1093/nar/gku555
- 187. De Luca, F. *et al.* Mutational analysis of single circulating tumor cells by next generation sequencing in metastatic breast cancer. *Oncotarget* 7, 1–11 (2016). DOI: 10.18632/oncotarget.8431
- 188. Peeters, D. J. E. *et al.* Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting. *Br. J. Cancer* **108**, 1358–67 (2013). DOI: 10.1038/bjc.2013.92
- 189. Morimoto, A. *et al.* High-density dielectrophoretic microwell array for detection, capture, and single-cell analysis of rare tumor cells in peripheral blood. *PLoS One* 10, (2015).
 DOI: 10.1371/journal.pone.0130418

- 190. Datta, S. *et al.* Laser capture microdissection: Big data from small samples. *Histology and Histopathology* 30, 1255–1269 (2015). DOI: 10.14670/HH-11-622
- 191. Mathiesen, R. R. *et al.* High-resolution analyses of copy number changes in disseminated tumor cells of patients with breast cancer. *Int. J. Cancer* 131, E405-15 (2012). DOI: 10.1002/ijc.26444
- 192. El-Heliebi, A. *et al.* Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer? *J. Transl. Med.* 11, 214 (2013). DOI: 10.1186/1479-5876-11-214
- 193. Ran, R. *et al.* Determination of EGFR mutations in single cells microdissected from enriched lung tumor cells in peripheral blood. *Anal. Bioanal. Chem.* 405, 7377–7382 (2013). DOI: 10.1007/s00216-013-7156-y
- 194. Srinivasan, M. *et al.* Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am.J.Pathol.* 161, 1961–1971 (2002). DOI: 10.1016/S0002-9440(10)64472-0
- 195. Carpenter, E. L. *et al.* Dielectrophoretic capture and genetic analysis of single neuroblastoma tumor cells. *Front. Oncol.* 4, 201 (2014). DOI: 10.3389/fonc.2014.00201
- 196. Wyatt, A. W. *et al.* Genomic Alterations in Cell-Free DNA and Enzalutamide Resistance in Castration-Resistant Prostate Cancer. *JAMA Oncol.* 2, 1598 (2016). DOI: 10.1001/jamaoncol.2016.0494
- 197. Wang, G. *et al.* Balanced-PCR amplification allows unbiased identification of genomic copy changes in minute cell and tissue samples. *Nucleic Acids Res.* 32, e76 (2004). DOI: 10.1093/nar/gnh070

- 198. Azad, A. A. *et al.* Androgen receptor gene aberrations in circulating cell-free DNA: Biomarkers of therapeutic resistance in castration-resistant prostate cancer. *Clin. Cancer Res.* 21, 2315–2324 (2015). DOI: 10.1158/1078-0432.CCR-14-2666
- 199. Navin, N. *et al.* Tumour evolution inferred by single-cell sequencing. *Nature* 472, 90–94 (2011). DOI: 10.1038/nature09807
- 200. El-Heliebi, A. *et al.* Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer? *J. Transl. Med.* 11, 214 (2013). DOI: 10.1186/1479-5876-11-214
- 201. Court, C. M. *et al.* Reality of Single Circulating Tumor Cell Sequencing for Molecular Diagnostics in Pancreatic Cancer. *J. Mol. Diagnostics* 18, 688–696 (2016). DOI: 10.1016/j.jmoldx.2016.03.006
- 202. Luo, J. Non-invasive actionable biomarkers for metastatic prostate cancer. *Asian Journal of Urology* **3**, 170–176 (2016). DOI: 10.1016/j.ajur.2016.09.003
- 203. Jung, R. *et al.* Detection of micrometastasis by cytokeratin 20 RT-PCR is limited due to stable background transcription in granulocytes. *Br. J. Cancer* 81, 870–873 (1999). DOI: 10.1038/sj.bjc.6690778
- 204. Thalgott, M. *et al.* Detection of Circulating Tumor Cells in Locally Advanced High-risk
 Prostate Cancer During Neoadjuvant Chemotherapy and Radical Prostatectomy.
 Anticancer Res 35, 5679–5685 (2015). DOI:
- 205. Polzer, B. *et al.* Molecular profiling of single circulating tumor cells with diagnostic intention. *EMBO Mol. Med.* **6**, 1371–1386 (2014). DOI: 10.15252/emmm.201404033

- 206. Kelvin, J. *et al.* Single cell analysis of AR N terminal, AR C terminal and the ARv7 splice variant in the CTCs of metastatic castration resistant prostate cancer (mCRPC) patients. *Cancer Res.* **75**, (2015). DOI:
- 207. Shaw, J. A. *et al.* Mutation analysis of cell-free DNA and single circulating tumor cells in metastatic breast cancer patients with high CTC counts. *Clin. Cancer Res.* clincanres.0825.2016 (2016). doi:10.1158/1078-0432.CCR-16-0825 DOI: 10.1158/1078-0432.CCR-16-0825
- 208. Islam, S. *et al.* Characterization of the single-cell transcriptional landscape by highly multiplex RNA-seq. *Genome Res.* **21,** 1160–1167 (2011). DOI: 10.1101/gr.110882.110
- 209. Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* 9, 171–181 (2014). DOI: 10.1038/nprot.2014.006
- 210. Picelli, S. *et al.* Smart-seq2 for sensitive full-length transcriptome profiling in single cells.
 Nat. Methods 10, 1096–1098 (2013). DOI: 10.1038/nmeth.2639
- 211. Ang, R. R. Spectral image cytometry for circulating tumor cell identification. (2017).
 doi:10.14288/1.0340303 DOI: 10.14288/1.0340303

Appendices

Appendix A Supplementary Information for Morphological Study of CTCs in Prostate Cancer

A.1 Patients Information Summary

All patients have metastatic castration resistant prostate cancer (mCRPC). There was no significant correlation between PSA level and size of CTCs.

D-434		PSA			Cell size in	Standard
ID	Age	level (µg/L)	mCRPC	naive	diameter (µm)	Standard deviation
1	69	28	Y	Y	8.84	2.50
2	77	156.4	Y	Y	7.05	1.67
3	62	21.1	Y	Y	7.54	2.15
4	67	456.9	Y	Y	7.16	1.47
5	77	184.7	Y	Y	6.90	1.85
6	62	21.85	Y	Y	7.85	1.62
7	68	160	Y	Y	8.67	2.23
8	72	53	Y	Y	7.11	1.61
9	71	2200	Y	Y	8.55	1.49
10	67	50	Y	Y	8.24	1.77
11	83	17.6	Y	Y	8.50	1.13
12	53	146	Y	Y	8.22	2.28
13	66	240	Y	Y	8.95	1.89
14	75	637.8	Y	Y	7.46	2.50
15	N/A	N/A	Y	Y	7.86	1.68
16	N/A	N/A	Y	Y	8.60	1.17

 Table A.8.1 Patients information summary

Appendix B Red Blood Cell Lysis Protocols

In order to increase the throughput of the device, RBC lysis step was introduced to concentrate the blood sample. Various RBC lysis kits were investigated to select the one with the best tumor cell recovery using the spiked sample: (1) EasySep kit for CD45 and GlyA depletion (StemCell Tech., #18259 and #18352), (2) Density gradient using Histopaque 1119 (Sigma), (3) RosetteSep kit for CD45 depletion (StemCell Tech., #15122), (4) RBC lysis buffer: Ammonium Chloride (StemCell Tech., #07800), and (5) RBC lysis buffer (gBioscience, (# 786-649).

B.1 EasySep Kit for CD45 and GlyA Depletion with HetaSep

Red blood cells and CD45 positive white blood cells were depleted using the EasySep kit (Stemcell Tech., #18259 and #18352) according to the manufacturer's protocol. Briefly, prestained 22RV1 cells were doped in 8 ml of whole blood, mixed with HetaSep solution (Stemcell Tech, # 07806, 1:5 ratio, v/v), and centrifuged at 90 xg at room temperature with the brake off for 5 min. The supernatant was collected and remaining red blood cells and CD45 positive white blood cells were depleted using Glyphorin A and CD45 magnetic particles. After magnetic incubation, the enriched cells were collected and number of 22RV1 cells was counted.

B.2 Density Gradient Centrifugation using Histopaque 1119

According to the manufacturer's protocol, blood samples doped with pre-stained 22RV1 cells were carefully layered onto the upper Histopaque 1119 medium in the SepMate tubes (Stemcell Tech., #85415) The tubes were then centrifuges at 700 xg for 30 min. Histopaque 1119 (Sigma, density = 1.119 g/mL) permits the separation of mononuclear cells and granulocytes from whole blood. Combined with SepMate, the enriched cells were easily collected by pouring off the upper

layer, containing the isolated nucleated cells. After centrifugation, enriched cell fraction, washed once with PBS, the number of 22RV1 cells were counted.

B.3 RosetteSep Kit for CD45 Depletion with Histopaque 1077

RosetteSep Human CD45 Depletion cocktail (StemCell tech, # 15122) was used to deplete both red blood cells and CD45 expressing white blood cells. Briefly, the RosetteSep reagent crosslinks multiple red blood cells and unwanted cells (e.g. CD45+ white blood cells) to increase the density of the unwanted cells. The RosetteSep reagent was added to 8 mL of spiked whole, followed by 20 min incubation at room temperature. After the incubation, the sample was layered with Histopaque 1077 (Sigma, density=1.077 g/mL) in the SepMate Tube, and centrifuged at 1200 xg for 10 min. The enriched cells were collected, washed once with PBS, and the number of 22RV1 cells were counted.

B.4 Red Blood Cell Lysis Buffer (Ammonium Chloride)

Red blood cell lysis buffer from StemCell Technology (# 07800) was added to the spiked whole blood sample (1:10 ratio v/v). The samples were incubated for 10 min on the rotating rack and centrifuged down at 400 xg for 5 min. After the first wash with PBS, there was significant amount of RBC left, interfering with cell counting. One more washing step was performed in order to count pre-stained tumor cells.

B.5 Red Blood Cell Lysis Buffer (gBioscience)

Red blood cell lysis buffer from gBioscience (# 786-649) was added to the spiked whole blood sample (1:3 ratio, v/v). The sample was incubated for 5 min on the rotating rack and centrifuged

down at 2500 xg for 5 min, and the supernatant was carefully removed. After the first washing, the red pellet was still visible. The second washing was required to count the pre-stained tumor cells doped into the blood. It is important to note that the red pellet do not affect the performance of cell separation using the microfluidic ratchet device. However, the sample was washed twice in order to count the pre-stained cells to measure the recovery rate.

Appendix C List of Reagents

C.1 Antibodies used in Immunofluorescence Staining

- 1) Alex Fluor 488-conjugated anti-pan CK (Cat#4523, CellSignaling)
- 2) APC –conjugated anti-CD45 (Cat#304011, Biolegend)
- 3) Alex Fluor 594-conjugated anti-EpCAM (Cat#7319, CellSignaling)
- 4) Alexa Fluor 555-conjugated anti-AR (Cat#8956, CellSignaling)
- 5) VECTASHIELD Antifade Mounting Medium with DAPI (Cat#H-1200, Vector Laboratories)

C.2 Common Reagents used in Immunofluorescence Staining

- 1) Paraformaldehyde (PFA), 4% in PBS (Cat#J61899, Alfa Aesar)
- 2) Phosphate-Buffered Saline (PBS), pH 7.4 (Cat#10010049, ThermoFisher Scientific)
- 3) Tween 20 (Cat#003005, ThermoFisher Scientific)

4) Bovine serum albumin (BSA) (Cat#9998, CellSignaling)