Multi-parameter Fluorescent Analysis of Magnetically Enriched Circulating Tumor Cells

Dissertation

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Abstract

Circulating tumor cells (CTC) have been proved related to cancer metastasis from the early stage of tumorigenesis. However, the mainstream recognition of CTC still stays within the traditional definition of CTCs, which is CD45 negative, Cytokeratin (CK) positive and Epithelial Cell Adhesion Molecule (EpCAM) positive, while various types of CTCs have been found in the blood of cancer patients in different stages. A new criterion of CTC is therefore necessary to be set up.

In this document, CD45-based negative immunomagnetic separation, a new methodology to isolate CTC, is reported to maximize the recovery of heterogeneous CTCs. CTCs from 120 patient blood specimens were isolated through this magnetic assembly with an average of 2.56 in log depletion and 77% in nucleated cell recovery rate. The 9-color flow cytometry plots of enriched cells show that the cell populations of CD45 positive or negative, CK positive, EpCAM positive or negative are significantly higher in patient blood specimens. Meanwhile, the patient survival is significantly related to CD45+/-, CK+ cells detected in the flow plot of the enriched cells.
The existence of heterogeneous CTCs shown in the four-color fluorescent immunocytochemical (ICC) staining demonstrates the necessity of the negative enrichment approach for CTCs. CTCs positive on CD45, epithelial markers, mesenchymal markers, human epidermal growth factor receptor (HER) family markers, Hedgehog (Hh) pathway markers and DNA damage marker, gamma-H2AX were found in the enriched cells from cancer patient blood specimens.

Besides the ICC staining, in this work, RNA / DNA in situ hybridization (ISH) were applied to enriched cells in CTCs. The DNA fluorescent in situ hybridization (FISH) was applied to detect the gene amplification of epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR). In DNA FISH, the amplification ratio can be precisely calculated by counting the target gene labels and the centromere labels of the corresponding chromosomes. CTCs with HER2 and EGFR genetic amplification 1-3 are presented in the enriched cells from patient blood. Meanwhile, the RNA ISH is applied on enriched cells to analyze the tumor-related markers in transcription level. All markers applied to fluorescent ICC staining are also tested with RNA ISH to study the difference of oncogene transcription and expression.
To eliminate the inconsistency from the heterogeneity of CTCs in parallel experiment, the combined RNA ISH and immunostaining methodology was developed to analyze the same CTCs on both transcription and expression levels. With quantum dots and Nuance multispectral camera, 6-color staining combined RNA ISH and immunostaining methodology was established. This combined staining technology was also extended to flow cytometry analysis in CTC research. Moreover, the combined staining can integrate the advantages of both RNA ISH and ICC staining, which facilitates human papillomavirus (HPV) analysis on CTCs. The HPV16 E6 and E7 were studied on CTCs from head and neck tumor patients (both pathological HPV positive and negative), in which HPV positive, CK positive or negative populations were analyzed.

This document aims to establish a comprehensive analyzing system for CTC isolation and multi-parameter characterization in genetic, transcription and expression levels. This document is also to explore the heterogeneity of CTCs in breast cancer and head / neck cancer with different markers.
Dedication

This document is dedicated to my parents and grandparents, who are always trying to understand what I am doing thousands of miles away from Hangzhou and supporting me unconditionally.
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Chapter 1: Introduction

1.1 Introduction to Circulating Tumor Cell (CTC)

Circulating Tumor Cell (CTC) is believed to be the primary tumor cell invading human circulatory compartment and thereby attains ready access to virtually all organs in the body (Gupta, 2009). Some research indicates that CTC is closely related to cancer metastasis, thus the number of circulating tumor cells in human circulatory system is an independent predictor of progression-free survival and overall survival (Cristofanilli, 2004 and 2005). Hence, the analysis of CTC is an integral part of cancer studies.

Traditionally, CTC is defined as a nucleated cell which is positive in Cytokeratin(CK) and negative in CD45 (Kagan, 2002). As CTC is mainly derived from tumor epithelial cells, the positiveness of the surface epithelial cell adhesion molecule (EpCAM) is also used as an important criterion to identify CTC (Swaby, 2011). Hence, the mainstream perspectives used to require that CTC should be CD45 negative, CK and EpCAM positive.
However, some researches indicate that determining tumor cells in blood with a certain criterion is not just a positive-negative decision, but a term of probability. By morphologically immunochemical staining, E. Borgen et al. obtained the following three populations: (1) Tumor cell: Cell cluster, obviously enlarged or atypical nucleus; (2) Probable tumor cell: No convincing hematopoietic cells characteristics, but morphology occasionally seen in false positive hematopoietic cells; (3) Non-tumor cells (Borgen, 1999). The researches on probable tumor cells imply that there is not a well-defined criterion to identify CTC and that the traditional criterion (CD45 negative, CK positive and EpCAM positive) may be flawed.

In additional to traditional markers, i.e. CK, EpCAM and CD45, multiple markers have been investigated on CTCs. Epithelial-mesenchymal Transition (EMT) markers, for example, were used to characterize CTCs and proved to be involved in the metastatic potential of CTCs (Kallergi, 2011). Meanwhile, some research reported CTCs negative on CK and EpCAM, which may challenge the traditional criterion for CTCs (Mikolajczyk, 2011). These researches show the multiple markers on heterogeneous CTCs, which are related to clinical condition and may contribute to the researches in cancer diagnosis and drug test. Therefore, new CTC separation and characterization methods are needed to fulfill the requirement of the analysis of CTCs on multiple
markers. In this dissertation, several CTC separation and characterization approaches will be reported to effectively enrich and characterize CTCs.

1.2 Separation Methodology of CTC

Generally, the concentration of CTCs is about one in $10^5$-$10^7$ nucleated cells in cancer patient blood. Therefore, multiple enrichment steps are necessary to fulfill the concentration requirement of subsequent characterization methods. Previously, the separation approaches of CTC were general divided into two groups: size-based, including filtration, density gradient method, OncoQuick and Rosette Sept; immunomagnetically based, including magnetic bead method, CellSearch or CTC chips (Alunni-Fabbroni, 2010).

Due to the heterogeneity of CTCs, sized-based approach may meet a challenge in the cut-off threshold, as the macrophage in normal blood can probably be regarded as CTC and left on the filter. Therefore, the sized-based approach may be less specific.

The immunomagnetic-based approaches can be divided into positive selection and negative selection. Serving as a typical product of positive selection approach, CellSearch,
the only FDA-approval test for CTC assessment, targets EpCAM as the only criterion to positively select CTCs from nucleated cells in patient blood. However, the expression of EpCAM varies among tumor types (van der Gun B, 2010). The EpCAM-based positive selection can thus omit some tumor-related cells, which is EpCAM low positive or negative. In this dissertation, an unbiased CTC separation method will be reported and applied in patient samples.

1.3 Characterization Methodology of CTC

Currently, clinical treatment decision is largely based on immunohistochemical staining of the solid tumor tissue. However, as CTCs have been demonstrated to be closely related to tumor progression and survival time, researchers have begun to seek the criterion for CTC quantification and characterization to determine cancer progression. Initially, the number of CTCs was determined by EpCAM-based method and correlated with patient condition. After the heterogeneity of CTC was reported, multiple cell characterization methodologies were applied on CTCs, including magnetic labeling, immunocytochemical staining, flow cytometry, single cell RT-PCR, nucleic acid microarray, nucleic in situ hybridization, etc. All these methodologies have merits and flaws, as discussed in the corresponding chapters. However, comparing with EpCAM-based method which uses
EpCAM as the only parameter to analyze CTC, all of these methodologies provide multi-parameter analysis on CTCs.

To fulfill the demand of increasing markers on circulating tumor cell, the number of parameter involved in one single CTC analysis also needs to be increased. However, in terms of labeling and detection, the steric hinderance and the spectrum overlap may hold back the extending of the maximum number of parameters in CTC analysis. Meanwhile, unlike tumor tissue, with respect to many proven tumor-related markers, Human Epidermal Growth Factor Receptor 2 (HER2) for example, CTC may have very low expression. A highly sensitive characterization technique is hence required to analyze CTCs.

In this document, several multi-parameter characterization techniques of CTC will be optimized in extending both the maximum number of parameters and detection sensitivity. With these techniques, multiple heterogeneous of CTCs will be reported found in patients’ blood. With the result of CTC quantification, further discussion between patient conditions and numbers of different CTCs will be held to emphasize the importance of CTCs in tumor progression.
1.4 Objective and Scope of this Study

The ultimate goal of this research is to develop a comprehensive method to analyze CTCs and discover the relation between the patient condition, therapy effectiveness and heterogeneous CTC quantity. To achieve this goal, the following specific objectives will be pursued.

1. Develop CTC immunomagnetic separation system. The reliability of our quadrupole magnetic sorting device (QMS) for CTCs needs to be verified with large amount of blood specimens. CD45-based negative depletion for CTC enrichment will be optimized. CTC chip served as single CTC separation will be built up and optimized.

2. Establish multi-parameter CTC characterization methodology at protein level. Up-to-9-color flow cytometry analysis protocol for CTC will be established. Meanwhile, up-to-4-color fluorescent immunocytochemical (ICC) staining pattern need to be optimized. 6-color ICC staining for CTC will be achieved with quantum dots and spectral deconvolution.
3. Apply RNA in situ hybridization (ISH) and DNA fluorescent in situ hybridization (FISH) with tumor-related sequences to analyze CTCs. Several tumor-related DNA FISH probes will be tested to verify the abundance of oncogenes in CTC. The protocol of multi-parameter analysis of RNA ISH for CTC will be established to test tumor-related gene expression. The combined RNA ISH and ICC staining will be developed to label the same CTC both on transcription and expression levels. With quantum dots and spectral deconvolution, this combined staining can be pushed towards 6 colors.

4. Use the cell characterization methodologies developed above to categorize and quantify CTCs. The results will be correlated to patient condition to estimate the effectiveness of chemotherapy.

1.5 Dissertation Structure

The chapter arrangement in this document is similar to the chronological order in which we developed CTC separation and characterization methodology.
In Chapter 1, the concept of CTC is introduced, as well as the importance of CTC research and the current divarication on the definition of CTC. Current CTC separation and characterization methods get summarized to bring up the necessity of our research. In the last part, the objectives of this research are listed.

In Chapter 2, current CTC separation progress will be reviewed and our CD45-based immunomagnetic negative enrichment will be introduced. The spiking test proved the efficiency of the separation device which is subsequently applied to more than 200 patient blood specimens.

In Chapter 3, the procedure of 9-color flow cytometry analysis will be described, in which multiple tumor-related markers were used to labeling CTCs. The numbers of events on flow cytometry corresponding atypical CTCs gated by CD45, CK, EpCAM status were well correlated with patient survival.

In Chapter 4, the protocol of 4-color fluorescent ICC staining will be established and applied on CTCs. Atypical CTCs will be reported that are found in the staining with a series of tumor-related markers. And the optimization process on this 4-color staining is to be discussed to observe weakly expressed markers.
In Chapter 5, RNA ISH will be used on CTC staining. The methodology of the combined RNA ISH and fluorescent ICC staining on the same cells will be developed both on slide staining and flow cytometry. With quantum dots and Nuance multi-spectral camera, the 6-color staining incorporating both RNA ISH and fluorescent ICC staining will be used on part of strongly expressed markers. The human papillomavirus (HPV) markers on head and neck tumor patients will be involved and discussed in all these tests.

In Chapter 6, DNA FISH will target human epidermal growth factor receptor (HER) family markers on CTCs with our optimized procedure. Normal cells and overexpressing cells on HER family markers will be compared quantitatively.

In Chapter 7, RNA microarray analysis will be applied on CTC to explore potential tumor related markers. Major microarray products will be discussed to fit the low amount of RNA in CTCs.

In Chapter 8, the future direction of CTC separation and characterization will be discussed, as well as the strategy for developing advanced multi-parameter analysis on both nucleic acid and protein levels.
1.6 Reference


Chapter 2: CD45-based Negative Immunomagnetic CTC Enrichment

2.1 Introduction

The primary concern in CTC analysis is the low concentration of CTCs. As stated in Section 1.2, the concentration of CTCs is about one in $10^5$-$10^7$ nucleated cells in cancer patient blood. In most recent CTC-related studies, multiple enrichment steps were applied. Starting from patient blood, the initial enrichment step must be high in throughput and recovery. The quadrupole magnetic separation device (QMS) we developed can process up to $10^8$ cells in 5 min. The QMS device employs negative depletion based on CD45, which ensures a high recovery in CTC research.

This chapter will introduce the enrichment approach with QMS and discuss the performance of QMS device. To determine the performance, an evaluation criterion will be set up, with which buffy coat and cell line spiking test will be employed. Patient blood specimen will also be applied on the device to isolate CTCs and determine the efficiency. Part of this chapter has been published as Wu Y, Deighan C, et. al. Isolation and analysis of rare cells in the blood of cancer patients using a negative depletion methodology.
2.2 Review of Immunomagnetic CTC Separation Methodology

The immunomagnetic separation approach for CTC can be divided by positive and negative methods, in terms of the labeling antibody. The positive method uses tumor-related markers, such as EpCAM, HER2 and CK, to label CTCs and sort CTC out with the magnetic property on the labeling antibody complex. The negative method usually applies CD45 and CD66b to remove leukocytes, so as to enrich CTCs.

2.2.1 Epithelial Marker Based Separation

The traditional definition of CTC is mostly about the positiveness in epithelial markers. Accordingly, in the early years, most CTC-based separation methods were developed on epithelial markers. CellSearch, as the first FDA-approved CTC separator, applies EpCAM as the only markers to separate out and quantify CTCs. The validity of CellSearch has been proven on cell line spiking experiment and breast cancer patient blood specimens drawn within 72h and stored in 4 °C (Riethdorf, 2007). Based on the
Kaplan-Meier estimates of probabilities of overall survival, the cancer patients with <5 CTCs per 7.5 ml blood detected by CellSearch have better cancer prognosis than those with >5 CTCs per 7.5ml blood (de Bono, 2008). With the CellSearch system, the CTC concentrations from most patient blood specimens are below 50 CTCs per 7.5 ml blood. (Pestrin, 2009).

However, some aberrant expressions of EpCAM, such as up-regulated, down-regulated or de novo expression were found in epithelial tumor cells (Trzpis, 2007). These aberrant expressions make the total expression of EpCAM vary depending on the type of cancer and the progression of carcinoma. For this reason, EpCAM cannot be exclusively used as separation criterion for CTC.

To avoid the bias of EpCAM-based CTC separation introduced by the complex expression of EpCAM, positive selection methods on other CTC-related markers were developed. CK-based CTC enrichment was reported on breast cancer patient and got an average of 86% in cell recovery comparing with the result from flow cytometry (Witzig, 2002). As an advance in CK-based separation, a combined CK-based and EpCAM-based approach was developed to sort out CK+EpCAM-, CK-EPCAM+ and CK+EpCAM+ cells (Deng, 2008). However, some recent report proved that tumor-related cells can be
negative on both CK and EpCAM (Mikolajczyk, 2011). Therefore, the positive separation methodology based CK, EpCAM or both may omit CTCs of this kind. Meanwhile, the CTCs after epithelial-mesenchymal transition (EMT) possess more mesenchymal features than epithelial (Yu, 2011). Most CTCs after EMT cannot be enriched by epithelial-based positive selections.

2.2.2 CTC Chips

CTC chip is an emerging research field to separate CTC efficiently. It is a microfluidic platform to selectively separate out CTCs. CTC chip initially uses EpCAM-based positive selection with about 50% in purity (Nagrath, 2007). A following research reported the practicability and superiority of CTC chips, which can isolate out an average of 132 CTCs per ml patient blood and support the subsequent tumor mutation test (Maheswaran, 2008). With the CTC chip, the chance to find a CTC in patient and the CTC concentration detected are all higher than those with previous CTC separation devices. Considering the heterogeneity of CTC and aberrant expression of EpCAM as described in the previous chapter, the CTC chip is also extended to HER2-based (Galletti, 2014).
To further increase the separation efficiency, our collaborator developed on-chip magnetic separation device, which is able to encapsulate single cell in droplet (Chen, 2013). To enrich heterogeneous CTCs, this magnetic device aims to sort CTCs based on several EMT and Human Epidermal Factor Receptor (HER) family markers.

2.2.3 Quadruple Magnetic Separation (QMS)

The QMS device was initially introduce by our lab based on the previous success in dipole separator. With a centrifugal magnetic field, the quadrupole magnets can generate a linear increment on the magnetic field when comparing with the constant magnetic field in the dipole magnets (Zborowski, 1999). This feature of quadrupole magnets assists the corresponding device to increase the resolution. Subsequently, the effectiveness of QMS flow sorter was proved on enriching T lymphocytes with a post-enrichment purity over 90% (Sun, 1998). The QMS device is then improved based on Sun’s setup and led to a dual-exit and dual-entrance design, which can enhance the cell post-enrichment purity and eliminate the fluidic effect on the cell magnetic separation (Chalmers, 1998). In this version of QMS device, the remaining of magnetically labeled cells in the main stream can be as low as ~5% and the purity of the magnetic cells in the separated stream can reach 99%, which obviously meet the demand for CTC detection.
From the beginning, our QMS device was designed for CD45-based negative separation of CTCs. Comparing with the positive methodologies based on a certain cancer-related marker, the CD45-based negative depletion can retain heterogeneous CTCs, instead of a certain type of CTC.

2.3 Material and Device

2.3.1 Cell line and Blood Resource

120 samples were collected from 71 breast cancer patients enrolled in an institutional review board (IRB) approved protocol. All patients gave their informed consent to participate in the study. Patients enrolled in this study must be histologically confirmed as breast cancer stages I–IV with either estrogen receptor (ER) positive or progesterone receptor (PR) positive tumors that are not HER2 overexpressing or ER/PR/HER2 non-overexpressing (triple negative). In stages I–III, patients are eligible if they have not received any treatment (including hormonal therapy) for breast cancer other than surgery prior to baseline measurement. In stage IV, eligible patients may be previously treated for metastatic disease \( \leq 2 \) systemic treatments (excluding hormonal therapy) and about to begin a new course of hormonal treatment or chemotherapy. For patients in stage I–III,
blood was collected prior to treatment on the first day of adjuvant/neo-adjuvant treatment, on day 1 of the second cycle of treatment, and then four weeks after the final dose of adjuvant treatment. For patients in stage IV, blood was collected prior to treatment on the first day of a new treatment, on day 1 of the second cycle of treatment, and then four weeks after the administration of the final chemotherapy/hormone therapy given up to the time of documented disease progression.

In addition, peripheral blood was also obtained from patients with a diagnosis of squamous cell carcinoma in head and neck undergoing surgical resection for their disease and who had not been previously treated for this disease. The procedure was IRB approved and informed, written consent was obtained from all subjects. Blood samples were collected from a venous line, either immediately prior to, and/or post, surgery and processed within twenty-four hours after procurement.

Normal blood was obtained through an IRB-approved purchase of source leukocytes from the American Red Cross (Columbus, OH).

Breast cancer cell lines, MCF-7 (HTB-22) and BT474 (HTB-20), tongue cancer cell line, SCC-4(CRL-1624) and pharyngeal cancer cell line, Detroit 562 were procured from
ATCC (Manassas, VA). Human papillomavirus (HPV) positive cell lines, UM-SCC47 and SCC90, were provided by Dr. Quintin Pan’s lab, Department of Otolaryngology, The Ohio State University. Hedgehog pathway active cell line, T47D, was provided by Dr. Majumder’s lab, College of Medicine, The Ohio State University. Melanoma cell line, F-01, was provided by Dr. Carson, College of Medicine, The Ohio State University. These cells were grown to mid-log phase in Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro) with 10% fetal bovine serum (FBS) (Invitrogen) and 1% nonessential amino acid (Cellgro) at 37 °C in 5% CO2 atmosphere. Cell lines were harvested by washing the adherent cells with phosphate buffer saline (PBS) and subsequently incubating with Accutase (Innovative Cell Technologies, Inc.) for 10 min at 37 °C to detach cells from T-flasks. Accutase was then neutralized with the culture medium before pelleting the cells at 350g for 5 min. Cells were resuspended in culture medium for downstream experiments. A summary of cell line is presented in Table 2.1.

If not otherwise notified, the sources of the patient blood specimens, health donor control and cell line are the same in the following part.
2.3.2 Buffers and Labeling Reagent

Two buffers were involved in the negative enrichment procedure. The 10X lysis buffer was mainly used as erythrocyte lysis. The labeling buffer was used in cell washing, re-suspension and storage. The recipes of these two buffers are listed in Table 2.2.

The labeling reagent was the EasySep™ Human CD45 Depletion Kit purchased from Stemcell Inc. The kit magnetically labels CD45 antigen via a tetrametric antibody complex (TAC) which links the dextran-coated magnetic nanoparticle and CD45 antibody. This immunomagnetic labeling kit involves two reagents: a CD45 TAC (Stem Cell Technologies) and dextran-coated magnetic nanoparticles (Stem Cell Technologies).

2.3.3 Quadrupole Magnetic Separator (QMS)

The magnetic separation assembly requires a quadrupole magnetic separator, a syringe pump, syringes and valves. As shown in Figure 2.1, syringe 1 and syringe 3 are 50ml syringes for washing buffer storage and flow-through collection; Syringe 2 is 10ml syringes for storing pre-enrichment cells. The pump was purchased from Harvard Apparatus. The detailed procedure will be introduced in Part 2.4.
The quadrupole magnetic separator consists of a design of steel and neodymium magnets which surrounds the central flow channel. Figure 2.2-2.3 presents a cut away, top view of the magnet assembly (Figure 2.2), as well as the simulated magnetic field gradients within the device (Figure 2.3).

Depending on the diameter of the channel, the QMS device can handle up to 20 million cells per min. The maximum flow rate in the channel is 5ml per min to ensure the consistent separation efficiency. The maximum cell concentration in Syringe 2 is 2 million cells per ml to avoid cell clustering.

2.4 Method

The negative immunomagnetic cell separation method consists of three parts: 1) red blood cell (RBC) lysis, 2) immunomagnetic labeling on CD45, and 3) negative magnetic enrichment. The flow chart of the overall process is presented in Figure 2.4. To separate out rare non-hematopoietic cells with high purity and low cell loss, the protocol described below is an attempt to minimize the number of cell processing steps while depleting the maximum number of leukocytes and recovering the maximum number of CD45 negative cells.
2.4.1 Red Blood Cell Lysis

To remove RBCs, a chemical lysis step was employed on the blood. Due to the RBC’s relative weakness to osmotic pressure compared to other cell types, concentrated ammonium chloride lysis buffer was used. Previous spiking studies have demonstrated the superior recovery of spiked MCF7 cancer cells by chemical lysis in comparison to the Ficoll-Hypaque density gradient separation technique (Miller, 2012).

Approximately 7–17 mL of peripheral blood were collected in Vacutainer tubes (BD Biosciences) for CTC enumeration and processed within 4 hours from blood draw. The lithium heparin coating is beneficial in preventing clumping of the white blood cells prior to analysis. The RBC lysis procedure was carried out as previously published (Miller, 2012). Briefly, the blood was mixed with 1X lysis buffer at a ratio of 1:20 to the total volume. After 5 min incubation, the cell lysate was centrifuged. With the supernatant discarded, the cell pellets were collected and washed with labeling buffer prior to immunomagnetic labeling.
2.4.2 Magnetic Labeling

After RBC lysis step, the cells were resuspended in labeling buffer and counted by hemocytometer. A desired amount of cells were aliquoted for storage before magnetic labeling. While the cell counting and aliquoting are not enforced, these steps assist in quality control and troubleshooting, and provide cells for pre-enrichment control in the subsequent staining steps.

A number of additional labeling schemes and commercially available antibody/magnetic particle combinations were screened, including CD45 MicroBeads (Miltenyi Biotec), primary anti-CD45-PE (Beckman Coulter) conjugated to secondary anti-PE-MicroBeads (Miltenyi Biotec), and CD45 Dynabeads (Invitrogen) (Yang, 2009). The CD45 TAC was selected on the basis of its high performance, both in the percentage of CD45+ cells labeled and the purity and recovery in subsequent magnetic enriching step. The high performance is thought to be due to the bifunctional structure of the TAC formed with the two-step protocol. The anti-CD45 antibody end of the TAC, unhindered by magnetic beads, has higher affinity and specificity to CD45+ cells (Zhang, 2006, Ralph, 1987). To reduce the cell loss, the cells proceed to magnetic separation immediately after the incubation with magnetic nanoparticles and any unbound magnetic particles were
removed during the magnetic separation step.

The detailed magnetic labeling procedure is as follows:

1. Centrifuge the cell suspension at 350g, 5 minutes. Remove the supernatant.
2. Add 0.5 μl CD45 TAC per million cells, pipette thoroughly to mix.
3. Incubate 30 minutes, room temperature, tapping every 5 minutes to mix.
4. Add 1 μl nanoparticles per million cells to the labeled cell suspension. Pipette to mix.
5. Incubate 15 minutes, room temperature, tap every 5 minutes to mix.
6. Add labeling buffer to bring the total volume to 5 ml.
7. Load the sample into the magnetic sorter and proceed to QMS separation.

2.4.3 Negative Enrichment

As shown in Figure 2.1, the cell suspension after magnetic labeling was initially loaded into Syringe 2. Gradually, the cell suspension flowed through the channel which is trapped within the magnetic assembly, and subsequently into Syringe 3. The magnetically labeled cells were left attracted in a radial direction on the inner side of the outer wall of the channel; the unlabeled cells were collected by Syringe 3. An optimized volumetric flow rate, balancing this magnetic attraction in the radial direction and the shear stress
created on the wall by the flowing fluid, is 5ml/min (Tong, 2007). The detailed protocol is given below:

1. Pass the sorting channel (PreCelleon, Columbus, OH) through the quadrupole magnet and connect it to Syringes 1 and 2

2. Fill Syringe 3 with 60 ml of labeling buffer and connect it to the bottom port of the channel

3. With the flow directed to Syringe 1, slowly fill the channel and Syringe 1 by manually infusing the 60 ml labeling buffer in Syringe 3 until Syringe 3 is empty

4. Connect Syringe 3 to the syringe pump (Harvard Apparatus)

5. Turn Valve 2 to allow the Syringe 2 to fill with 5 ml labeling buffer, then set Valve 2 to connect Syringe 2 and the sorting channel

6. Load the sample (5 ml suspension) from the top of the Syringe 2

7. Start the syringe pump, set to refill 45 ml at 5 ml/min

8. When the pump has finished running, close Valve 3 and disconnect Syringe 3 from the sorting channel

9. Optional: If recovery of the deposited cells (those trapped in the sorting channel) is desired, Syringe 1 should be refilled with approximately 35 ml labeling buffer. A fresh syringe should be attached to Valve 3. By sliding the sorting channel out of the
magnet and rapidly filling the new Syringe 3, the cells in the sorting channel will be swept and collect in the new Syringe 3

10. Centrifuge the collected cell suspension at 350 x g for 5 min to pellet the cells.

11. Discard the supernatant with leaving approximately 0.5 ml labeling buffer. Resuspend the cells and count cells with hemocytometer to determine the number of the isolated cells.

2.4.4 Performance Evaluation

While this optimized protocol attempts to reduce the variability of the process, we observed a significant variability in the performance of the removal steps of RBCs and peripheral blood lymphocyte (PBL). We typically measured the numbers of the nucleated cells before RBC lysis, after RBC lysis, and after magnetic separation as the parameters for enrichment performance evaluation.

To obtain nucleated cell counts, the cell suspensions were added to 3% acetic acid at a ratio of 1:25 for before-RBC-lysis and after-RBC-lysis cells, and a ratio of 1:10 for the cells after magnetic enrichment. After 10 min incubation at room temperature, the cell number was counted by Fuchs-Rosenthal hemocytometer (Hausser Scientific). Incubating
with 3% acetic acid can dramatically reduce the background interference caused by unlysed RBCs, cell debris and other contaminants.

As a measure of the overall depletion process performance, the total numbers of PBLs in the initial blood sample, after RBC lysis, and after magnetic separation and the total number of cells in the blood were enumerated. With these cell counts, RBC lysis efficiency, nucleated cell log depletion and total cell log depletion were calculated as parameters for performance evaluation.

The RBC lysis efficiency was used to evaluate the PBL recovery in the RBC lysis step as Eq. 2-1.

\[
Lysis
efficiency = \frac{N_{\text{after lysis}}}{N_{\text{before lysis}}} \times 100\% \quad (2-1)
\]

\(N_{\text{before lysis}}\) and \(N_{\text{after lysis}}\) are the number of nucleated cells before and after lysis, respectively.

The nucleated cell log depletion was used to evaluate the efficiency of the CD45-based magnetic negative depletion and calculated as Eq. 2-2.

\[
\text{nucleated cell log}_{10} \text{depletion} = \log_{10} \left( \frac{N_{\text{before depletion}}}{N_{\text{after depletion}}} \right) \quad (2-2)
\]
\( N_{\text{before depletion}} \) and \( N_{\text{after depletion}} \) are the number of nucleated cells before and after magnetic depletion, respectively.

The total cell log depletion was used to evaluate the efficiency of the overall non-hematopoietic cell enrichment by comparing the total number of cells in the blood to the number of cells left after depletion as Eq. 2-3.

\[
\text{total cell } \log_{10} \text{ depletion} = \log_{10} \left( \frac{N_{\text{total blood}}}{N_{\text{after depletion}}} \right)
\]  
(2-3)

\( N_{\text{total blood}} \) is the total number of cells in the blood.

### 2.4.5 Cell Storage

Cells were aliquoted in labeling buffer from the cell suspension before magnetic labeling and after magnetic sorting. In order to preserve cells for future analysis, the cells before or after negative magnetic enrichment were usually stored in RNAlater (Ambion) or 70% ethanol (EtOH). The cells stored in RNAlater were reserved for future nucleic acid analysis, while the cells stored in 70% EtOH with 4% paraformaldehyde fixation were reserved for immunofluorescence staining.
The cells to be stored in RNAlater were first washed with 1 ml 1X PBS and then centrifuged for 5 min at 350g. With supernatant discarded, RNAlater was added to the cell pellets by 100 ul per million cells. The cells were stored at 4 °C for the first 24 hours and then transferred to −20 °C or −80 °C for long term storage. The RNA can remain intact at −20 °C for up to a year and at −80 °C for more than a year.

The cells to be stored in 70% ethanol were first washed with PBS and centrifuged for 5 min at 350g. With supernatant discarded, cells were fixed with 1 ml 4% paraformaldehyde per million cells for 10 min. After centrifugation, the supernatant was removed and 1 ml 70% EtOH per million cells is added. The cells in 70% EtOH should be stored at −20 °C and valid for 2 years.

2.5 Result and Discussion

2.5.1 Immunomagnetic Separation Performance

The immunomagnetic separation step discussed above uses a highly optimized magnetic and fluidic assembly which imposes on the cell suspension a magnetic gradient on the order of 300 T/m, as well as the hydrodynamic forces which significantly reduce the
non-specific binding of cells to the inner surfaces of separation channel. The magnetic depletion is accomplished as the cell suspension flows through an open annular channel trapped within a very high magnetic gradient. The magnetic pressure used to hold the magnetically labeled cells against the inner wall of the annulus is sufficient to allow cell suspension flowing through the annulus in a relatively rapid rate. The fairly high flow rate not just increases the cell process rate, but also lowers the chances that unlabeled cells are retained within the annulus.

To measure the performance of the negative immunomagnetic separation, the results of 120 breast cancer patient samples were analyzed, as well as 9 head and neck cancer patient samples. Both the total cell counts and nucleated cell counts of the initial samples were recorded, as well as nucleated cell counts after both RBC lysis and magnetic depletion. Eq. 2.1–2.3 were used to determine the performance of the RBC lysis and the magnetic depletion, while Eq. 2-2 was used to calculate the nucleated cell log depletion, thus to determine the specific performance of the quadrupole magnetic separator. And Eq. 2-3 was used to calculate the total cell log depletion, which includes the performance of the RBC lysis. For the breast cancer patient samples in this study, the average nucleated cell log depletion is 2.56, while the average total cell log depletion is 5.63. Therefore, on
average, only 0.28% and 0.0002% of nucleated cells and total blood cells remain after depletion.

Since the immunomagnetic separation described here is based on the depletion of hematopoietic cells, it is important to consider not only the number of cells removed at each step, but also the number of cells of interest that are retained. For the RBC lysis, the objective is to remove RBCs, but it is also important to retain as many nucleated cells as possible. A measure of the performance on cell retaining can be calculated from Eq. 2-1, the lysis efficiency. The average lysis efficiency for the breast cancer patient set analyzed here is 77%. For the magnetic depletion, the analogous performance measure is CTC recovery. However, in patient samples, it is impossible to know with certainty the initial number of CTCs. An estimation of this performance based on spiking studies will be presented in the next section.

### 2.5.2 Recovery of spiked cancer cell lines

A common challenge with respect to evaluating the performance of rare cell isolation technologies, such as the separation/recovery of circulating tumor cells, is to find the proper criteria and systems. Unfortunately, in many cases, the numbers and types of rare
cells in actual human samples are unknown. Therefore, spiking studies are commonly used, such as spiking cancer cell line cells into normal blood. In the spiking test, the cancer cell lines are assumed in the same behavior as CTCs. Nevertheless, the morphologies and characteristics of cell line cells and CTC may be different, thus the behaviors of cancer cell line and CTC can possibly be different.

Figure 2.5 is a log-log plot of the number of cancer cells recovered versus the number of cancer cells initially spiked into normal blood (the types of cancer cells are listed in the legend). This data was obtained over a six-year period, with four different cancer cell lines and five different researchers in our group. All numbers are reported as the number of cancer cells per ml of blood equivalent (a value of $6 \times 10^6$ nucleated cells per ml of blood was assumed, which is consistent with the data observed with cancer patient blood samples). The lower plot is an enlarged view of a portion of the plot above to show better detail in the range of 10–100 cells per ml blood equivalent. The dark, solid line corresponds to a 100% recovery. Linear regression lines through the origin are presented for two cell lines: MCF7 and SCC4. Regression analysis of the MCF7 data (light, solid line) produces a slope of 0.39 (95% CI: 0.37–0.42) and correlation coefficient ($R^2$) of 0.99. Analysis of the SCC4 data (dash line) produces a slope of 0.84 (95% CI: 0.83–0.85).
and an $R^2$ of 1.00. The other cell lines, F-01 and Detroit 562, were tested at a single spiking level, with which linearity studies were not performed.

It is important to note the relatively high level of performance observed with the SCC4, F-01 and Detroit 562 cell lines (~80% recovery) compared to the poor recovery of MCF-7 (~45%). A number of speculations can be made for the poor performance of MCF-7, including significant clumping which compromises accurate cell counts. However, such variation with respect to different cell lines underscores the care that must be taken in interpreting and comparing spiking studies.

### 2.6 Conclusion

In this chapter, our CD45-based negative enrichment methodology for CTCs is introduced. Comparing with prevailing positive enrichment, our method can retain heterogeneous CTCs, which will be presented in the following chapters. Meanwhile, unlike positive CTC separation, the CTCs enriched from our negative depletion are not labeled with any antibodies or magnetic particles, which possibly influence the subsequent analysis. The effectiveness of the QMS device has been proven by the ability to remove 99.72% hemopoietic nucleated cells in more than 200 patient blood specimens.
from 120 breast cancer patients. In our negative enrichment system, CD45 can be switched to any other hemopoietic cell markers to further enrich CTCs, such as CD66b and CD4. To increase the selective accuracy, CTC chip may be a direction for the development of CTC enrichment. The on-chip CD45-based negative CTC enrichment has been developed recently (Karabacak, 2014). A CTC chip with higher throughput capacity and accuracy is under development in our lab.

2.7 Reference


• Chen A, Byvank T, et al. On-chip magnetic separation and encapsulation of cells in droplets. Lab Chip (2013);13, 1172-1181.


• Pestrin M, Bessi S, et al. Correlation of HER2 status between primary tumors and


- Yang L, Lang J, et al. Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal


<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue of Origin</th>
<th>Morphology</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>Luminal A</td>
<td>CK8,18,19++, EpCAM+, E-CAD+, HER2+, CK5,6-, CD45-</td>
</tr>
<tr>
<td>BT474</td>
<td>Breast</td>
<td>Luminal B</td>
<td>HER2++, EGFR+, EpCAM+, E-CAD+, CK8,18,19+, CK5,6-, CD45-</td>
</tr>
<tr>
<td>T47D</td>
<td>Breast</td>
<td>Luminal A</td>
<td>Gli++, Smo++, CK8,18,19+, CK5,6-, CD45-</td>
</tr>
<tr>
<td>SCC-4</td>
<td>Tongue</td>
<td>Basal</td>
<td>EFGR++, CK5,6++, HER2+, CK8,18,19+, CD45-</td>
</tr>
<tr>
<td>SCC90</td>
<td>Oral</td>
<td>Basal</td>
<td>HPV16++, EFGR+, CK5,6+, CK8,18,19+, CD45-</td>
</tr>
<tr>
<td>UM-SCC47</td>
<td>Tongue</td>
<td>Basal</td>
<td>HPV16+, EFGR+, CK5,6+, CK8,18,19+, CD45-</td>
</tr>
<tr>
<td>Detroit 562</td>
<td>Pharyngeal</td>
<td>Basal</td>
<td>Not used as control in this paper</td>
</tr>
<tr>
<td>F-01</td>
<td>Melanoma</td>
<td>Basal</td>
<td>Not used as control in this paper</td>
</tr>
</tbody>
</table>

Table 2.1 Summary of tumor cell lines and respective control markers
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysis Buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Ammonium Chloride</td>
<td>20.1g</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>2.1g</td>
</tr>
<tr>
<td>EDTA, tetrasodium salt dihydrate</td>
<td>0.093g</td>
</tr>
<tr>
<td>ddH2O</td>
<td>to 250ml</td>
</tr>
</tbody>
</table>

| **Labeling Buffer**    |            |
| Phosphate Buffer Saline (PBS) | 1L         |
| bovine serum albumin,(BSA)   | 5g         |
| EDTA, tetrasodium salt dihydrate | 0.74g |

Table 2.2 Recipe of lysis buffer and labeling buffer in cell separation
Figure 2.1 Picture of Quadruple Magnetic Separator
Figure 2.2 The cross section of the magnetic assembly of QMS

Purple = steel
Yellow = neodymium magnets

(Dimensions in inches)
Figure 2.3 The simulated magnetic field gradients within QMS device
Figure 2.4 The flow chart of CTC separation and characterization with QMS
Figure 2.5 Log-log plot of the number of cancer cells recovered versus the number of cancer cells initially spiked in the spiking test for QMS performance.
Chapter 3 Flow Cytometry Analysis on CTCs from Metastatic Breast Cancer Patient

3.1 Introduction

Although more than 99% nucleate cells have been removed from patient blood, the CTC concentration from the enriched cells after the CD45-based negative depletion described in the previous chapter is still low. The average cell number after the negative enrichment is about $10^4$-$10^5$ per ml blood. Flow cytometry can provide fast and accurate sorting and analysis for the cell number in this order of magnitude.

Previously, multi-color flow cytometry was used to analyze CTCs on chemokine receptors (Fusi, 2012) and CKs (Cruz, 2005). However, with more tumor-related markers studied on heterogeneous CTCs, the increasing requirement to distinguish CTC subpopulations needs more parameters or colors in a single flow cytometry analysis. In this chapter, 9-color flow cytometry analysis will be introduced on negatively enriched cells with optimized antibody selection. Enriched cells from the blood specimens of 32
breast cancer patient samples will be tested to study the impact of certain tumor-related markers on patient survival time. The CD45 optimization part is cited from our previously published paper: Wu Y, Deighan C, et al. Isolation and analysis of rare cells in the blood of cancer patients using a negative depletion methodology. *Methods.* (2013); 64(2):169-82. And the patient study part is cited from our recently published paper: Lustberg M, Balasubramanian P, *et al.* Heterogeneous atypical cell populations are present in blood of metastatic breast cancer patients. *Breast Cancer Res.* (2014); 16(2):R23. The patient clinical data collection and analysis in Figure 3.2-3.4 and Table 3.4 is the contribution of Dr. Maryam Lustberg and Dr. Brandon Miller

3.2 Material and Device

3.2.1 Patient Specimen and Healthy Control

Blood specimens of 32 metastatic breast cancer patients were selected from the 120 patients introduced in 2.3.1. These 32 metastatic breast cancer patients involve three kinds of breast cancer in terms of the status of HER2, estrogen receptor (ER) and progesterone receptor (PR). The patients positive on HER2 but negative on ER were classified as HER2+; the patients positive on ER but negative on HER2 were classified as
ER+; the patients negative on all these three markers were classified as triple negative breast cancer (TNBC). In these 32 patients, 15 are TNBC; 14 are ER+; 3 are HER2+.

Healthy donor blood was collected from volunteer donors (n=8) after obtaining informed consent using an IRB-approved protocol at The Ohio State University Medical Center and processed in the same manner as the patient samples.

3.2.2 Conjugated antibody and labeling reagent

Three kinds of labeling reagents were applied in the flow cytometry staining analysis. The cell viability status was determined by LIVE/DEAD Fixable Aqua Dead Cell Stain Kit or LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies). The antibodies for surface markers, including macrophage markers CD115 and CD14, natural killer cell marker CD16 and CD56, hemopoietic markers CD45, epithelial marker EpCAM and HER2, were used. The antibodies for intracellular markers, including epithelial marker CK and macrophage marker CD68 were also used in the 9-color flow analysis. The detailed information of antibodies is listed in Table 3.1. OneComp eBeads (ebioscience) and ArC™ Amine Reactive Compensation Bead (Molecular Probes) were used as compensation controls for antibodies and LIVE/DEAD Fixable dyes, respectively.
3.2.3 Flow Cytometer

Two flow cytometers located in the Analytical Cytometry Shared Resource at The Ohio State University, were used in this study. All analysis-only experiments were conducted on a BD FACS LSR II instrument with the FACS Diva software for data acquisition and analysis. All cell sorting and analysis experiments were performed on a BD ARIA III flow cytometer. The flow cytometers were calibrated using cytometer setup and tracking (CST) and DNAQC beads in the CST module in the Diva software. Automatic compensation was performed according to Diva protocol with unstained and single-color controls with Onecomp / ArC beads. At least 10,000 events were recorded per sample for analysis. FlowJo 10.0.6 (Tree Star) was used to set the population gates and analyze the data.

The BD FACS LSR II flow cytometer was equipped with three lasers: violet laser(407nm), blue laser (488nm) and red laser (633nm). The BD ARIA III flow cytometer was equipped with 4 lasers: UV laser (355nm), blue laser (488nm), Yel-GR laser (561nm) and red laser (640nm). The filters and corresponding fluorochromes are listed in Table 3.2.
3.3 Method

The labeling procedure began with viability marker, followed by surface antibody binding. After fixing, permeabilizing and blocking, the cells would be processed by intracellular markers. To prevent the fluorochromes from bleaching, the whole procedure was supposed to be carried out in the dark. The detailed procedure is as follows:

1. Wash the enriched cells from Chapter 2 with 1 ml PBS, centrifuge with 350 g, 5 min and discard supernatant

2. Add viability dye from LIVE/DEAD Fixable Aqua/Blue Dead Cell Stain Kit, 1ul per million cells

3. Incubate at room temperature for 30 min, in the dark

4. Wash the cells with 1 ml PBS, centrifuge with 350 g, 5 min and discard supernatant

5. Upon research need, add part of or all surface markers listed below to cells. The amount of each antibody is 5ul per million cells

Abs: CD115-PE, CD45-PE-Cy7, EpCAM-APC, CD16-AF700, CD56-ECD (PE-Texas Red)

6. Incubate at room temperature for 30 min, in the dark

7. Wash the cells with 1 ml PBS, centrifuge with 350 g, 5 min and discard supernatant

8. Add 200ul BD Cytofix/Cytoperm Fixation and Permeabilization Solution to fix and
permeabilize cells

9. Incubate at 4 °C for 10 min, in the dark

10. Wash the cells with 1 ml 1X BD Perm/Wash Buffer, centrifuge with 350 g, 5 min and discard supernatant

11. Add 1 ml 1% FBS in PBS to block

12. Incubate at room temperature for 30 min, in the dark

13. Centrifuge with 350 g, 5 min, discard supernatant

14. Add part of or all following intracellular markers into tubes

   Abs: CK-FITC 10 μl / million cells, CD68-PerCP-Cy5.5 5ul / million cells

15. Add 20ul 0.5% Saponin

16. Incubate at room temperature for 30 min, in the dark

17. Wash the cells with 1 ml PBS, centrifuge with 350 g, 5 min and discard supernatant

18. Add 300 μl Sorting Buffer (Table 3.3) to each tube and store at 4 °C for flow analysis

In the flow protocol above, if HER2 primary antibody was applied in the surface marker antibody step, 1ul of donkey anti-rabbit Alexa Fluor (AF) 555 (Invitrogen) should be added to label HER2 primary antibody after Step 7. And a 30min incubation step in dark and a wash step were required before fixation step.
For LIVE/DEAD Fixable Aqua/Blue dye, 10ul ArC beads (Component A) and 10ul ArC beads (Component B) were used in the viability single-color control for compensation. Meanwhile, for all other antibodies, each control used 20ul OneComp eBead solution. Comparing with the antigens on cells, these beads have higher binding affinity towards antibody. In order to apply the same laser voltage on the stained cells and single-color control, the antibodies for these single-color controls need to be diluted. For each single color control, the antibody amount for 20ul beads is as follows:

- LIVE/DEAD Fixable Aqua Dead Cell Stain Kit - 1 μl;
- CD115-PE – 1/8 μl;
- CD45-PE-Cy7 – 1/16 μl;
- EpCAM-APC – 1/8 μl;
- CD16-AF700 – 1 μl;
- CD14-APC-AF750 – 1 μl;
- CK-FITC – 1/8 μl;
- CD56-ECD – 1 ul;
- CD68-PerCP-Cy5.5 – 1/8 μl.

During the flow cytometry procedure, gates were set on forward scattering (FSC) and side scattering (SSC) by comparing with the unstained cell controls. These gates aim to exclude cell debris and keep singlets for analysis. A viability gate was also set based on the viability control so as to exclude dead cells.
3.4 Result and Discussion

3.4.1 Statistical Significance of Rare Event in Flow Cytometry

Given the rarity of CTCs, the numbers of CK+ or EpCAM+ events are possibly as low as 10-1000 per ml blood. The threshold of statistical significance on the number of the events is therefore crucial in this study. Assuming the number of events in a certain subpopulations in replicates follows Poisson distribution, the coefficient of variation (CV) can be calculated as follows:

\[ CV = \frac{1}{\sqrt{r}} \]  \hspace{1cm} (3-1)

Here \( r \) is the number of events detected in a certain subpopulation.

Some literature uses CV=10% as the threshold to determine the counting error in breast cancer progenitor cell analysis (Zimmerlin, 2011). In the following research, we set two threshold levels on CV for counting error: 3% and 10%. The approximate corresponding event numbers are 1000 and 100.
3.4.2 Distinct Subpopulations of Atypical Cells

Despite the >99% of CD45+ nucleated cell removal in the negative depletion step before the flow cytometry, the typical outcome in the flow analysis still includes quite a number of CD45 low positive cells. Figure 3.1 shows the flow result of an enriched patient sample, in which 18.4% cells are CD45 low positive. Different atypical subpopulations of cells are found in the patient sample, such as the CK+, CD68+, CD16+, CD14+ population which has both features of epithelial cells and macrophage. At this section, the cells either positive on CK or EpCAM will be in the research scope.

For 18 TNBC patients, plots of the different subpopulations of the enriched patient samples in terms of the number of events per ml of blood sample, as identified on flow cytometry, are presented in Figure 3.2. For comparison, these same subpopulations are also presented for the healthy donors. As shown in Table 3.4, the numbers of CD45+ CK+ EpCAM-, CD45+ CK+ EpCAM+, CD45- CK+ EpCAM-, and CD45- CK+ EpCAM+ cells in patient samples are all statistically higher than those in healthy control samples (p=0.03, 0.0007, 0.0004 and 0.0008 respectively). Regardless of EpCAM status, the numbers of CK+ CD45- and CK+ CD45+ events are also statistically higher in patient samples than in control samples (p=0.004 and 0.028, respectively). Inconsistent
with the generally accepted concept that the concentration of the traditionally defined CTCs is not high enough to be routinely detected in flow cytometry analysis without sampling large blood volumes (i.e. greater than 20 mL), the CD45- CK+ EpCAM+ population concentrations are significantly larger than the control; however, most of the concentrations are below the 100 events per ml of blood.

Additional comparisons of the subpopulations presented in patient samples highlight that there are significantly more CK+EpCAM- events than CK+EpCAM+ events in both CD45- and CD45+ fractions (both p<0.0005). We observed similar elevated populations of CK+EpCAM- (both CD45- and CD45+) in the metastatic ER positive, and the HER2 positive patient populations. The EpCAM- events are not statistically different between the ER+ versus TNBC patients (Figure 3.3).

3.4.3 Clinical Outcome Correlations

Combining all three types of metastatic breast cancer, we studied whether elevated concentrations of these sub-types are predictive of progressive free survival (PFS) or overall survival (OS), as determined by a Kaplan-Meier (KM) estimator analysis. As a
binary cut-off for the KM analysis, we used the criteria that the targeted population had to exceed a specific concentration. For a CV of 5%, 400 events are needed. Given our smallest blood sample of approximately 4.5 ml, this corresponds to a concentration for the CD45- CK+ EpCAM- population to be on the order of 100 events per ml of blood. For the CD45+CK+EpCAM- population, we used the mean plus one standard deviation of the normal patient population as a cut-off. These four KM plots are presented in Figure 3.4.

3.4.4 Selection of CD45 Antibody Clones Using Flow Cytometry

According to the flow plot in Figure 3.1, there are many CD45 low positive cells in the enriched patient sample, which may not be able to bind the sufficient number of magnetic beads and remain in the flow-through. From the previous section, along with CK and EpCAM, CD45 status of these cells is also a significant parameter related to patient survival. Considering the steric hindrance between CD45 antibodies used in cell separation and flow cytometry staining, the CD45 antibodies in flow analysis need to be optimized. Other than MEM-28 which is the CD45 clone in the TAC for negative depletion, six CD45 clones were tested as listed in Table 3.5. The after-magnetic-labeling
(AML) and before-magnetic-labeling (BML) cells were stained with CD45 antibodies and then flowed.

Similar to the patient samples, the flow data from all magnetic labeled or unlabeled cells were also first gated on FSC and SSC to remove cell debris and select singlet events, followed by gating on the viability dye based on the results from the unstained tube to select only viable cells. All remaining events were used for further quantitative analysis of the CD45 clones. Figure 3.5 presents flow cytometry histograms of the six anti-CD45 antibody clones from seven manufacturers tested in the CD45 flow analysis. The unstained tubes were used to set the threshold between negative and positive events in each case. Based on these results, a majority of AML events from the clones of F10-89-4\_NB, 5B1, 2D1 and HI100 are negative, while the BML events of these clones are positive. The AML curve of clone J.33 is partially shifted but remains positive. The AML curves of clone HI30 and F10-89-4\_AbD are only slightly shifted.

To further facilitate the interpretations of these histograms, after multiple repeats, Figure 3.6 presents the percentage of cells considered positive for each antibody clone. A good clone, which is suitable for use in analysis applications to identify CD45 positive cells in the enriched fraction, should not only stain majority of leukocytes, but has little change in
the percentage of positive cells between the BML and AML groups. Meanwhile, given that CD45 is a pan-leukocyte marker, we would expect greater than 97% of the cells should be positive (Ralph, 1987). Therefore, a good CD45 antibody clone for labeling should be positive on at least 97% of AML cells.

In our study, significant variation between clones can be observed with respect to the fraction of the cells that are positive for CD45. Although most clones are effective for staining the BML fraction, in the AML staining, the incompatible clones fail to provide an adequate number of positive cells, for example F10-84-9_NB, which gives no positivity at all.

These results indicate that for our specific study, only clones J.33, HI30, and F10-89-4_AbD are acceptable, for the staining is not inhibited when the cells are previously labeled with the CD45 TAC (clone MEM 28). By identifying these compatible clones, potential staining inconsistencies leading to false negative results for CD45 on the isolated cell fraction can be eliminated.
3.5 Conclusion

With the 9-color multi-parameter analysis, Atypical CTCs are found among negatively enriched cells from metastatic breast cancer patients. While the number of CD45-, CK+, EpCAM- cells is significantly related to patient survival from KM plot on OS and PFS, the number of CD45+, CK+, EpCAM- cells indicates a relationship to patient survival. In terms of EpCAM+ cells, the multi-parameter flow analysis shows the difference between TNBC patients and healthy donors.

More subpopulations can be explored in 9-color flow test to find tumor-related markers. In our system, there are 36 subpopulations by gating with 2 markers; and each subpopulation can be divided by 4 parts based on the positiveness of these 2 markers. Meanwhile, 84 subpopulations can be obtained by gating with 3 markers; and each subpopulation can be divided by 8 parts. Therefore, rigorous statistical analysis should be conducted to explore new cell subpopulations related to tumor progression. CD16, CD68 and CD115 are good candidates to be researched on, since reports on these macrophage markers have shown their association with tumor progression.
3.6 Reference

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugated fluorochrome</th>
<th>Clone</th>
<th>Host</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>APC-AF750</td>
<td>TüK4</td>
<td>mouse</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD16</td>
<td>AF700</td>
<td>3G8</td>
<td>mouse</td>
<td>Invitrogen</td>
</tr>
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<td>CD45</td>
<td>PE-Cy7</td>
<td>HI30</td>
<td>mouse</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-Texas Red</td>
<td>N901 (NHK-1)</td>
<td>mouse</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
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<td>Biolegend</td>
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</tr>
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<td>29D8</td>
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<td>Cell Signaling</td>
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* HER2 is a pure antibody and needs to be labeled with Alexe Fluor(AF) 555 in the staining step

Table 3.1 List of antibodies used for flow cytometry immunostaining
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<th>Laser</th>
<th>Dichroic</th>
<th>Band Pass</th>
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<tr>
<td>PE</td>
<td>488nm</td>
<td>550LP</td>
<td>575/26</td>
</tr>
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<td>PE-Texas Red</td>
<td>488nm</td>
<td>600LP</td>
<td>610/20</td>
</tr>
<tr>
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<td>488nm</td>
<td>685LP</td>
<td>710/50</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>488nm</td>
<td>735LP</td>
<td>780/60</td>
</tr>
<tr>
<td>APC</td>
<td>633nm</td>
<td>-</td>
<td>660/20</td>
</tr>
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</tr>
<tr>
<td>APC-Cy7</td>
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<td>735LP</td>
<td>780/60</td>
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<table>
<thead>
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<td>488nm</td>
<td>505LP</td>
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<td>685LP</td>
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<tr>
<td>PE</td>
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<td>610/20</td>
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<td>640nm</td>
<td>690LP</td>
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<tr>
<td>APC-Cy7</td>
<td>640nm</td>
<td>750LP</td>
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Table 3.2 Configuration of flow cytometer for immunostaining
Table 3.3 Recipe of sorting buffer for flow cytometry

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<th>Quantity</th>
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<tr>
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<tr>
<td>EDTA Solution (0.5M, pH 8.0)</td>
<td>2ml</td>
</tr>
<tr>
<td>NaOH (1N)</td>
<td>5ml</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>0.5g</td>
</tr>
<tr>
<td>Hank's Balanced Salt Solution</td>
<td>to 500ml</td>
</tr>
<tr>
<td>Subpopulation</td>
<td>CD45-CK+ EpCAM+</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Overall</td>
<td>27.6 (4.5, 72.3)</td>
</tr>
<tr>
<td>Baseline (n=9)</td>
<td>16 (1.3, 50.4)</td>
</tr>
<tr>
<td>Progression (n=9)</td>
<td>72.3 (9.8, 139.6)</td>
</tr>
<tr>
<td>Control (n=8)</td>
<td>1.1 (0, 8.6)</td>
</tr>
<tr>
<td>P Values</td>
<td>0.01</td>
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The values in each cell are Median (Q1, Q3). p-values are from the Mann-Whitney U tests of the subpopulations between control and the patient samples.

Table 3.4 Mann-Whitney U tests of the cell subpopulations in flow plots
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugated fluorochrome</th>
<th>Clone</th>
<th>Host</th>
<th>Manufacturer</th>
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<td>PE</td>
<td>J.33</td>
<td>mouse</td>
<td>Immunotech</td>
</tr>
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<td>CD45</td>
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<td>F10-89-4</td>
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<td>Novus Biologicals</td>
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<td>APC</td>
<td>F10-89-4</td>
<td>mouse</td>
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<td>2D1</td>
<td>mouse</td>
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<td>CD45RA</td>
<td>APC-H7</td>
<td>HI100</td>
<td>mouse</td>
<td>BD Pharmingen</td>
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Table 3.5 Clones of CD45 in binding test on magnetically labeled cells
Figure 3.1 A typical flow plot of the 9-color staining on the enriched cells from cancer patient blood
Figure 3.2 The number of events in the different subpopulations of the enriched TNBC patient and healthy donor samples in flow cytometry analysis.

Figure 3.3 The number of events of CK+ subpopulations in the enriched cells from TNBC, ER+, HER2+ patient and healthy donor.
Figure 3.4 Kaplan-Meier plot of Progress-free Survival and Overall Survival for the patients with 100 +/- CD45-, CK+, EpCAM- events/ml and 1000 +/- CD45+, CK+, EpCAM- events/ml
Figure 3.5 Flow cytometry curves of the seven anti-CD45 antibody clones on AML, BML and unstained cells
Figure 3.6 Statistical Histograms of the seven anti-CD45 antibody clones on AML, BML
Chapter 4 Multi-parameter Fluorescent Immunocytochemical Staining for CTC Characterization

4.1 Introduction

Immunocytochemical (ICC) staining is the most prevailing approach to analyze CTCs. In an ICC staining, a primary antibody selectively binds to the corresponding antigen on the cell surface or inside of the cell. Subsequently, a secondary antibody with labeling probes will be used to bind the primary antibody. Generally, the labeling group on the secondary antibody can be radioactive element, enzyme, regular color dye or fluorophore. Within these labeling groups, fluorophore is most commonly used for the convenience, harmlessness and high resolution. In the progress in developing the QMS device, the evaluation of the CTC separator was partly based on ICC at the early stage (Tong, 2007). The ICC staining was then applied with multiple markers on the enriched patient specimens from QMS (Balasubramanian, 2012; Garcia-Villa, 2012).
In this chapter, fluorescent immunocytochemical (ICC) staining on CTCs with multiple surface, intracellular and intranuclear markers is presented. The fluorescent staining protocol was optimized to provide a higher specificity and to reduce the spectral overlap in multi-color staining. The usage of several kinds of microscopes will be discussed to reach the highest detection threshold and highest numbers of parameters in analysis. Part of this chapter has also been published as Wu Y, Deighan C, et al. Isolation and analysis of rare cells in the blood of cancer patients using a negative depletion methodology. *Methods.* (2013); 64(2):169-82.

4.2 Review on Fluorescent Immunocytochemical (ICC) Staining Technique for CTCs

4.2.1 Fluorescent Probe for CTC ICC Staining.

Unlike tumor tissue staining, in a majority of the cases, the tumor-related markers are only weakly expressed in CTCs. The staining methodology on CTC requires sufficient amplification to exceed the minimum detectable threshold for the specific microscope. Therefore, regular labeling antibodies for bright field or dark field may not be eligible for CTC staining. Meanwhile, the heterogeneity of CTCs requires CTC characterization at the single cell level. Given the high amplification of signals, cell-based ELISA, which
detects the overall signal of all the cells inside of a plate well, is thus not an optimal choice for the CTC staining.

Fluorescent ICC staining has been widely used to analyze CTC since the milestone report in which novel CTC separator was developed (Nagrath, 2007). Since the morphology or certain antigen expression levels of individual cells can be viewed with several labeling markers at the corresponding fluorescent channels, the fluorescent ICC staining can identify the heterogeneity of single CTCs. Previously, the applicability of some fluorochromes was controversial due to the bleaching issue. The signals from two common fluorescent probes, R-PE and FITC, need to be corrected for the bleaching problem (Schauenstein, 1980; Greenberg, 1993). Some tandem dyes, including APC-Cy7, PerCP-Cy5.5 and PE-Cy7, were also found degradation in the fixing, low temp storage, and light exposure (Le Roy, 2009). To increase the stability of fluorescent probes, some small molecular fluorescent probes were developed and commercialized, such as Alexa Fluor (AF) from Invitrogen, Chromeo from Active Motif, HiLyte Fluor from AnaSpec, etc. Researches showed that these probes have significant higher resistance from photo-bleaching and emit more intense fluorescent signals (Mahmoudian, 2011; Berlier, 2003).
To further improve photostability and brightness to capture the weak expression of tumor related proteins, quantum dots (QD) were applied into immunocytochemical staining. Quantum dots are nanoscale material made of semi-conductor. While the emission from organic fluorescent probes is based on the electron migration among the energy levels of the conjugated π bond, which may deteriorate by structure alteration or dissociation when exposed to intense light; the emission from QD is based on the relaxing of excited electron towards ground state in a semi-conductor, which is very stable under laser illumination. And the superior photostability is proven practically by parallel experiment against Alexa Fluor on Ig G and streptavidin conjugated antibody (Montón, 2009).

The band gap energy of QD is reversely related to the size. As a result, the emission frequency of QD is highly tunable, for the emission will shift from blue to red when size increases. Meanwhile, the excitation peak of most QDs is below 400nm. Therefore, the excitation and emission wavelengths of QD are very different from those of organic fluorochromes, which make QD good complements or substitutes in multi-parameter analysis.

However, since the size of QD is generally larger than organic fluorochromes, intensive permeablizaion is required for intracellular marker staining with QD. As the emission
frequency is reversely related to size, only QD with high emission frequency can be applied to intracellular markers (Xing, 2007). To penetrate cell membrane to label intracellular markers, peptide coating on QD is required for improving staining efficiency (Liu, 2010). Moreover, QD without special coating may aggregate or bind non-specifically to cell membrane. To reduce non-specific binding, coating with bovine serum albumin (BSA) or hydroxyl-derivation has been studied (Zhang, 2012; Kairdolf, 2008). In a word, special treatment of QD is usually necessary to optimize staining result.

4.2.2 Microscopy Device

To observe the fluorescent staining on CTC, confocal microscopy system is commonly used. By point illumination and pinhole design, confocal microscope is able to detect the fluorescent intensity emitted at a certain pixel, excluding the signals out of focal plane. Thus, confocal microscope can provide 3D images in CTC analysis, by which the antigen bound to fluorochromes can be allocated. As the confocal microscope forms image by horizontal pixel scanning, the exposure time for the confocal microscope on each pixel must be short to take an image within a reasonable duration. However, for the weakly expressed antigens on CTCs, the laser intensity for excitation must increase to keep the exposure time practical. However, as stated in Section 4.2.1, most organic fluorochromes
can be bleached under laser above certain intensity. Meanwhile, in the CTC imaging, to shorten the exposure time with relatively low laser intensity, the number of pixel scanned is low. As a result, the resolution of CTC image from confocal is not very high.

Epifluorescence microscopy system is the conventional fluorescent microscope equipped with an arrangement of filters which can split the excitation illumination and emission signals. The filter set for epifluorescence microscope consists of three parts: excitation filter, dichronic filter and emission filter. The excitation and emission filters are usually band-pass filters, while the dichronic filter is long-pass. The light with the wavelength shorter than the cut-off of the dichronic filter can be reflected, while the light with longer wavelength can penetrate the filter. The broadband light initially passes through the excitation filter, gets reflected on the dichronic filter and excites the fluorochromes on cells. The emission light, which has longer wavelength, is able to pass through the dichronic filter and then excitation filter. Comparing with the confocal microscope, epifluorescence microscope does not scan by pixel, thus requires a shorter exposure time, which can reduce photobleaching (Korlach, 1999). Moreover, as the epifluorescence microscopy obtains the signals from all pixels at the same time, the exposure time for a certain pixel can be 100-fold higher than confocal. Therefore, epifluorescence microscope has a more powerful ability to detect weak signals in CTC staining.
Nuance Multispectral camera (Abbr as Nuance) can scan image data at a certain wavelength across near infrared, near ultraviolet and visible spectrum. In Nuance, there are multiple sets of tunable filters controlled by voltage. The superposition of passbands of these tunable filters can create a very narrow passband at a certain wavelength, which allows Nuance to collect images at every 10nm in wavelength. The chief benefit with Nuance usage is the ability to remove spectral overlapping among different fluorochromes. In order to reverse spectral overlapping, the emission intensity curves from every single fluorochrome are measured first with single color staining slides. Then Nuance scans all wavelengths of the multi-color stained patient cell slides with different excitation wavelengths. With the emission intensity curves of single fluorochrome, Nuance software can deconvolve the signals from all the wavelengths to calculate out the emission intensities from every single fluorochrome marker. Because Nuance scans the whole spectrum instead of a certain range, unlike differentiating emission signal by filter set, Nuance is not bothered by spectrum leaking.

In this and next chapter, all of these three microscopy systems will be applied to CTC fluorescent staining and characterize CTCs with respective advantages.
4.3 Markers in CTC Characterization

4.3.1 Hematopoietic Marker

CD45 is a common marker for nucleated hematopoietic cells. A CD45 negative cell has been widely accepted to be a feature of CTC and some non-leukocyte rare cells in blood (Armstrong, 2011). Therefore, CD45 should always be one of the markers on the staining of rare cells in patient blood. As discussed in Section 3.4.4, CD45 antibody clone HI30 has been tested to ensure reliable staining results with our depletion system and is the only clone used for the staining presented in this discussion.

4.3.2 Epithelial Markers

Although not all epithelial cells in human blood necessarily originate from a tumor site (Goeminne, 2000), the positive expression of epithelial markers is used as an important criterion to identify CTCs (Swaby, 2011). In most CTC research, CK and EpCAM are used as two typical markers for epithelial cells. CK is a family of proteins shaping the intermediate filament in the cytoskeleton of epithelial tissue or cell. At least 29 kinds of proteins are in CK family. In most of adenocarcinoma cells, some simple cytokeratins, such as CK 8, 18, 19, are usually expressed, while CK7 and CK20 are variably expressed
depending on the type of cancer (Karantza, 2011). As a major type of adenocarcinoma, breast cancer usually expresses CK7, 8, 18, 19 and selectively expresses CK5,6; for example in basal-like breast cancer. For that reason, we use the anti-CK 8,18,19 for our CK staining studies.

EpCAM, a second key epithelial marker, is a pan-differential antigen that was initially found as a marker for tumor cells which express EpCAM in a higher level than normal epithelial cells. Previous research shows that the CD45 negative, CK positive and EpCAM positive cells are found in the magnetic enriched cells from patients with squamous cell carcinoma of head and neck (Balasubramanian, 2012).

However, some aberrant expressions of EpCAM, such as up-regulated, down-regulated or de novo expression were found in epithelial tumor cells (Trzpiz, 2007). These aberrant expressions make the total expression of EpCAM vary depending on the type and the progression of carcinoma. For this reason, EpCAM cannot be exclusively used as the epithelial marker of interest. This argument can be supported by the CK and EpCAM staining of the magnetically enriched cells from metastatic breast cancer patient’s blood, as discussed in Chapter 2.
4.3.3 Mesenchymal Markers

Epithelial mesenchymal transition (EMT) has been reported to exhibit dynamic changes within the progression of breast cancer and chemotherapy (Yu, 2013). Some research claims that CTCs can be CK 8,18,19 negative and EpCAM negative during or after the EMT (Mikolajczyk, 2011). Fluorescent ICC staining with mesenchymal markers is therefore necessary to identify these CTCs which do not conform to the traditional definition.

Vimentin is a type of intermediate filament protein and consistent with mesenchymal cells. In the cells undergoing EMT, without changing the other markers of EMT, vimentin induces the alteration of cell shape, adhesion and motility (Mendez, 2010). It is widely used as a marker to study epithelial and mesenchymal protein co-expression on CTCs in the phenotypic state of EMT (Sieuwerts, 2009; Armstrong, 2011).

Cadherin can mediate cell adhesion with the regulation of calcium ion. Within the cadherin family, N-cadherin(N-CAD) and E-cadherin(E-CAD) are major regulators of adhesion and integrity of epithelial cells. However, research shows among cancer cells, the vimentin-positive cells are more likely to acquire mesenchymal properties, even
before the switch from E-CAD to N-CAD, thus the frequency of expression of vimentin in breast tumors is markedly higher than N-cadherin as an EMT marker (Sarrió, 2008). Improved understanding of their expression patterns will give valuable insight into this dynamic.

4.3.4 Human Epidermal Growth Factor Receptor (HER)

Human Epidermal Growth Factor Receptor (HER) family includes four members: EGFR/HER1, ErbB2/HER2, HER3, and HER4. The signaling cascades related to proteins in the HER family have important prognostic and predictive significance in a variety of solid tumors and have several targeted therapies that are currently approved or in development. Therefore, the characterization of CTCs on these drugable targets is of significant clinical interest since CTCs may give the most dynamic snapshot of the state of tumor progression. EGFR and HER2 are hence useful markers to identify rare cells in cancer patient blood.

EGFR is a cell surface marker that induces cell differentiation and proliferation and is activated by 7 growth factors, including epidermal growth factor and transforming growth factor α. The proportion of the expression of EGFR in breast, lung and colon
cancer cell is 55%-60%, which is relatively high, comparing with the expression of 15%-30% for HER2 and 20%-50% for HER4 (Normanno, 2006). HER2 is considered as the key role in HER signaling. HER2 is a preferred dimerization partner for EGFR. And the major function of HER2 is to amplify EGFR signal (Zhang, 2009).

4.3.5 Other markers of emerging interest-CD44, Gli, Smo, γ-H2AX

Malfunction on Hedgehog (Hh) pathway can lead to increased cell proliferation and tumor formation (Rubin, 2006). In CTCs, Hh signaling pathway is abnormally active and positively related to the invasiveness of CTCs (Shaw, 2008). Therefore, markers of the Hh pathway are one of the potential labeling targets for diagnosis and drug inhibition. The overexpression of Gli1, the effector protein in Hh pathway, has significant effects on down-regulation of E-CAD, thus transforms human epithelial cells into an invasive phenotype (Feldmann, 2007). Research shows that in neoplastic cells, the expression of Gli1 is independent from the regulation of Smoothened (Smo) which is a G-Protein coupled surface receptor in Hh pathway in human epithelial cells. (Nolan-Stevaux, 2009) The Gli1 and Smo staining is hereby an important part to identify CTCs, of which Hh pathway is irregularly activated.
Cancer stem cells are tumor-forming and tumor-maintenance cells, performing cell differentiation and proliferation into different tumor cell types. As a major hyaluronan receptor, CD44 is considered to be involved in homing, settling and differentiating cancer stem cells (Zöller, 2011). CD44 knockdown can inhibit tumorigenesis, tumor cell migration and invasion. (Hiraga, 2013)

γ-H2AX, which is phosphorylated H2AX on Ser 139, is a sensitive target for the recognition of DNA double stand breaks. The foci from the γ-H2AX staining on tumor cells are essential for the evaluation of chemotherapy. In this report, γ-H2AX staining on CTC will be used as a criterion to determine the effectiveness of ABT-888 treatment.

4.4 Material and Equipment

4.4.1 Immunofluorescence staining antibody

Various antibodies targeting cellular proteins relevant to CTCs, including extracellular and intracellular markers, are presented in Table 4.1. In addition to the typical markers used to detect CTCs, i.e., DAPI, CK, CD45, and EpCAM, additional targets were
selected based on review of current literature, interest from colleagues in clinical oncology, and relevance to cancer-related therapies, as described in Section 4.3.

The primary antibodies were fluorescently labeled using the secondary antibodies in Table 4.2. In addition, two of the most commonly-used primary antibodies were custom-conjugated directly to fluorescent dyes: CK-AF488 (clone: CK3-6H5) and CD45-AF594 (clone: HI30).

4.4.2 Microscopy System

In this chapter, two microscope systems were used to analyze the stained slides and capture images: a Nikon Eclipse 80i epifluorescence microscope with Intesilght C-HGFi mercury vapor lamp and DS-Qi1Mc digital CCD camera, and an Olympus FV1000 spectral confocal system. The Nikon epifluorescence microscope was equipped with the filter sets in Table 4.3 for detection of four-color staining with DAPI, FITC or AF488, AF555 or AF594, and AF633 or AF647. The Olympus FV1000 spectral confocal system, located in the Campus Microscopy and Imaging Facility at The Ohio State University, was equipped with four lasers, a mercury vapor lamp, two spectral detectors, two
filter-based detectors, and a transmitted DIC detector. Table 4.4 lists the configuration used for detection of the four-color staining of the confocal system.

### 4.4.3 Cell Line and Blood Source

The source of cell lines and blood specimens is the same as introduced in Section 2.3.1

### 4.5 Method

The immunofluorescence staining protocol has been demonstrated to work well to identify rare cells in the blood of cancer patients. Up to four fluorescent colors can be used simultaneously on the same slide. The staining protocol began with 1000rpm centrifuge on cytospins. Each slide may contain up to 20,000 cells. For normal leukocytes and cell line controls, the cell concentration was counted and 20,000 cells were added onto each slide. For the enriched peripheral blood samples of cancer patients, the cells cytospun onto slides included rare non-hematopoietic cells, e.g., CTCs, as well as leukocytes that were not removed during the enrichment process due to low expression of CD45. For this reason, most staining protocols include DAPI, CK, and CD45 for CTC recognition, as well as one additional marker for characterization.
The protocol began by fixing the cells with 4% paraformaldehyde, followed by permeabilization and blocking with normal serum blocking solution (NSBS) (Table 4.5). Cells were then incubated with primary antibodies before the treatment with the corresponding secondary antibodies. Slides were subsequently mounted with ProLong Gold Antifade Reagent with DAPI and sealed with nail balm. The following protocol shows an example using CK-AF488, CD45-AF647, gammaH2AX-AF594 and DAPI staining (Garcia-Villa, 2012).

1. Fix cells with 4% paraformaldehyde for 10 min.

2. Permeabilize and block cells with NSBS for 30 min.

3. Add 100 μl diluted anti-CD45 (1:100 dilution) and anti-gammaH2AX (1:100 dilution) primary antibodies in antibody diluent (MP Biomedicals) and incubate for one hour.

4. Wash the slides three times with PBS containing 0.05% Tween-20 (PBST) for 5 min each.

5. Add 100 μl diluted goat anti-mouse AF594 (1:400 dilution) and donkey anti-rabbit AF647 (1:400 dilution) secondary antibodies in PBST and incubate for one hour in the dark.

6. Wash the slides three times with PBST for 5 min each.

7. Add 100 μl diluted CK-AF488 custom-conjugated antibody (1:100 dilution) in PBS containing 1% bovine serum albumin (BSA) and incubate for one hour in the dark.
8. Wash the slides three times with PBST for 5 min each.

9. Mount coverslip with ProLong Gold Antifade Reagent with DAPI.

The example shown above can be generalized when using unconjugated primary antibodies requiring the addition of secondary fluorescent dyes and pre-conjugated antibodies. All steps were performed at room temperature. Steps 5 – 8 can be skipped if all antibodies used are conjugated to fluorescent dyes, while steps 9-10 can be skipped if no pre-conjugated antibodies are used. When primary antibodies are applied in the staining, different host species should be used for each primary antibody to prevent cross reaction of secondary antibodies. The dilution factor varies due to the different concentrations provided by antibody manufacturers, but the optimal final concentration is typically 5-10 μg/ml for primary and conjugated antibodies and 1-5 μg/ml for secondary antibodies. In all cases, the antibodies are titrated using appropriate controls to determine the optimal dilution.

4.6 Result

As discussed in Section 3.4.2, the result from flow cytometry shows the numbers of various types of CK or EpCAM positive cells (CD45+CK+EpCAM-,
CD45+CK+EpCAM+, CD45-CK+EpCAM-, and CD45-CK+EpCAM+) significantly increase in TNBC patients. To visualize the result, four color fluorescent ICC staining was initially tested with DAPI, CK, EpCAM and CD45. Figure 4.1 presents the result of four color staining and microscopy analysis, of a TNBC patient sample enriched in the manner presented in Chapter 2 along with control samples. In Figure 4.1, (a)-(e) are leukocytes, (f)-(j) are breast cancer cell line MCF7, and (k)-(o) are cells from an enriched peripheral blood sample from a metastatic breast cancer patient. The first column is a combined image of the four colors and the remaining columns are DAPI, anti-CK-AF488, anti-CD45-AF594, and anti-EpCAM-AF647, respectively. For the patient sample, Cell #1 is CK+ CD45+ EpCAM- and Cell #2 is CK- CD45+ EpCAM+. The patient sample demonstrates that rare non-hematopoietic cells are not always consistent with the stereotype that both CK and EpCAM markers present on the same cell.

The progression of EMT is usually evaluated by a pair of epithelial and mesenchymal markers, such as CK/vimentin and N-CAD/E-CAD. Variations in CK/vimentin expression level are observed between different cells from the same patient. Figure 4.2 shows an enriched cell from patient blood with immunofluorescence staining of CK and vimentin. In Figure 4.2, (a)-(e) are leukocytes, while (f)-(j) are the cells from an enriched peripheral blood sample from a cancer patient. The columns (left to right) are DAPI,
anti-CK-AF488, anti-CD45-AF594, anti-VIM-AF647, and a combined image of the four colors, respectively. The patient cell in Figure 4.2 appears CK positive, CD45 negative, VIM weakly positive, thus can be considered undergoing the EMT process. With the addition of N-CAD, Figure 4.3 presents some patient cells that are positive on CK, N-CAD and vimentin. The staining setting of CK, N-CAD and vimentin can be used in further EMT research to identify the cells undergo EMT and to analyze the EMT progress on tumor cells. If additional markers are needed, either N-CAD or vimentin can be taken out from the setting in Figure 4.3 and vacate the channel for the additional markers. Figure 4.4 shows a patient cell under EMT (CK+ and VIM+), as well as EGFR positive.

Beyond the typically reported epithelial and mesenchymal markers discussed above, a range of other markers have elicited interest in the rare cell analysis field. Cells positive on some of these markers are found in the enriched cells from cancer patient blood specimens. These markers include HER2 (Figure 8), Smo (Figure 9), Gli1 (Figure 10), and a cellular response to DNA damage marker, γ-H2AX. Figures 4.5-4.7 are the staining of enriched nucleated cells from metastatic breast cancer patient. Figure 4.5 is the result of 4-color staining with DAPI, CK, CD45, and HER2. The cell in this figure is CK+ CD45- HER2+, thus a HER2-expressing CTC. Figure 4.6 is the result of 4-color staining with DAPI, CK, and Smo. The three cells in the yellow boxes are CK+ Smo+, thus Hh
pathway active CTCs. Figure 4.7 is the result of 2-color staining with DAPI and Gli1. In Figure 4.7, a Gli+ cell and a Gli- cell are present in the scope. All γ-H2AX-related are the work of Garcia-Villa A (Garcia-Villa, 2012). Therefore, γ-H2AX staining is not presented in this document.

4.7 Discussion

4.7.1 Atypical Cancer-related Cell

In this chapter, several atypical cancer-related cells are identified by the 4-color fluorescent ICC staining. In the staining of CK, EpCAM and CD45, the recognition of CK+ CD45+ EpCAM+ and CK+ CD45+ EpCAM- cells supports the result from Section 3.4.2. Considering the significance between survival time and CK+, EpCAM+/– cell occurrence, as shown in Figure 3.3 and 3.4, the quantification of cell numbers on fluorescent ICC slides can assist prognosis. The vimentin+ and N-CAD+ cells are found in metastatic breast cancer patient. These cells under EMT are probably negative in EpCAM, hence have a high chance to be neglected by EpCAM-based positive separation methodology. Fluorescent ICC staining is the major analytical method for CTCs under EMT (Lecharpentier, 2011).
Number of CTCs positive in HER2 is significantly related to breast cancer survival (Liu, 2013). The CK+, CD45-, HER2+ cells found in patient enriched cells may provide prognostic information for therapy design. Overexpression of EGFR is related to occurrence head and neck squamous tumor, for aggressive head and neck tumor is usually positive (Thariat, 2012). Therefore, the EGFR+ CTC number can estimate head and neck tumor progress. Meanwhile, Smo, Gli and CD44 are major chemotherapy drug targets. CTC staining on these markers can contribute to evaluate the effectiveness of chemotherapy.

4.7.2 Antibody Choice for Immunofluorescence Staining

With the same antibody clone, in terms of binding specificity, antibodies pre-conjugated to fluorescent dyes perform better than the combinations of unconjugated primary antibodies and secondary fluorescent dyes. In each staining, only one primary antibody from each given host can be applied. In spite of additional blocking step, significant non-specific binding will appear if two unconjugated primary antibodies from the same host species are added onto the same immunofluorescence staining slide. However, if the primary antibody is already conjugated with secondary antibody, two or more primary
antibodies from the same host species can stain the same slides without significant non-specific binding. For uncommon antibodies, some protein labeling kits are successfully applied by previous research (Karadag, 2004), and are preferred to minimize non-specific binding.

4.7.3 Device and Color Choice for fluorescence ICC staining

The excitation and emission curves for fluorescent dyes are not sharp peaks on light wavelength chart, but can have, relatively speaking, large ranges in both excitation and emission. Therefore, using two fluorescence dyes, which have close emission or excitation spectrum (usually within about 50nm), can lead to spectrum overlapping and thus false positivity. Figure 4.8 is excitation and emission spectra of AF555 and AF594, showing the relative excitation and emission intensities when excited at 562 nm and detected at 625 nm, respectively. Despite the 50nm gap at the peak value between excitation and emission curves, significant overlapping still exists. The argument is also supported by some researches (Schieker, 2004). Due to the spectrum overlapping and false positivity issues, few researches show five-or-more-color fluorescence ICC staining on the same cells. A good setting for 4 colors to split and use whole spectrum can be AF405 or DAPI, FITC or AF488, AF555 or AF594, and AF633 or AF647.
For five-or-more-color fluorescence ICC staining, QD together with Nuance may be a solution, since the excitation wavelength of all QDs is below 400nm and hence far away from those of most secondary fluorescent dyes, while the emission wavelength of all the quantum dots is near those of most secondary fluorescent dyes and far away from DAPI’s (Byers, 2011).

Mercury bulb is a common illuminating source for fluorescence. However, the emission spectrum of mercury bulb is not constant. To obtain a good view, the antibody with weaker binding to the target cells or the antibodies corresponding to lowly expressed antigens should be conjugated to the fluorescent dye whose excitation peak locates at one of the emission peaks of mercury bulb. On the other hand, to eliminate the interference towards the neighbor fluorescence channel, the antibodies with stronger bindings to the target cells or the antibodies corresponding to highly expressed antigens should use fluorescent dye whose excitation peak locates at relatively low part of the emission curve of the mercury bulb. In our 4-color-split staining, AF405, AF555 and AF594 are better choices to show antibody with weaker binding, since their excitation peaks partially overlap with the emission peaks of mercury bulb. However, the AF488 and AF 647 are suitable for antibody with stronger binding to target cells (Figure 4.9). If all antibodies
applied in a single staining have weak binding to the target cells, xenon lamp or laser illuminator should be good choices.

4.8 Conclusion

Our 4-color fluorescent ICC staining provides a feasible and visible method to identify rare CTCs in CTC-enriched cancer patient blood cells. This staining can bring a clear view of different cell markers (both for surface and intracellular markers) with relatively minimum spectrum overlap and interference. And the success in identifying rare CTCs with nucleic acid dye, CD45, epithelial markers, mesenchymal markers, HER markers, Hh pathway markers and cancer stem cell markers, provide a visible way to do multi-parameter analysis on counting various types of CTCs. Further research can be made with the correlation between the characteristics or numbers of a certain type of cells in patient blood and medical records, such as patient condition, treatment, drug test, etc. The visibility makes the counting on fluorescent ICC staining slides more reliable than flow cytometry. More parameters are expected to be put on the same cells with recently developed fluoroprobes and fluorescence detectors.
4.9 Reference


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<table>
<thead>
<tr>
<th>Target</th>
<th>Clone</th>
<th>Host species</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus (DAPI)</td>
<td>-</td>
<td>-</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CK 8, 18, 19</td>
<td>CK3-6H5</td>
<td>mouse</td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>CD45</td>
<td>HI30</td>
<td>mouse</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD45</td>
<td>(polyclonal)</td>
<td>rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>EpCAM</td>
<td>(polyclonal)</td>
<td>rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>EGFR</td>
<td>(polyclonal)</td>
<td>rabbit</td>
<td>Abcam</td>
</tr>
<tr>
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<td>29D8</td>
<td>rabbit</td>
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<td>Abcam</td>
</tr>
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<td>Abcam</td>
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<td>D-1</td>
<td>mouse</td>
<td>Santa Cruz Biotech.</td>
</tr>
<tr>
<td>Smo</td>
<td>(polyclonal)</td>
<td>rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>JBW301</td>
<td>mouse</td>
<td>Millipore</td>
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Table 4.1 Primary antibodies in fluorescent ICC staining on slides
Table 4.2 Secondary antibodies in fluorescent ICC staining on slides

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Target (IgG)</th>
<th>Host species</th>
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<tr>
<td>AF488</td>
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<td>goat</td>
</tr>
<tr>
<td>AF555</td>
<td>anti-goat</td>
<td>donkey</td>
</tr>
<tr>
<td>AF594</td>
<td>anti-mouse</td>
<td>goat</td>
</tr>
<tr>
<td>AF633</td>
<td>anti-rabbit</td>
<td>donkey</td>
</tr>
<tr>
<td>AF647</td>
<td>anti-goat</td>
<td>donkey</td>
</tr>
<tr>
<td>AF647</td>
<td>anti-rabbit</td>
<td>donkey</td>
</tr>
</tbody>
</table>

Table 4.3 Filter sets for Nikon epifluorescence microscope

<table>
<thead>
<tr>
<th>Filter set</th>
<th>Excitation</th>
<th>Dichroic</th>
<th>Emission</th>
<th>Fluorescent dyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>377/50 BP</td>
<td>409</td>
<td>447/60 BP</td>
<td>DAPI</td>
</tr>
<tr>
<td>Green</td>
<td>475/50 BP</td>
<td>506</td>
<td>536/40 BP</td>
<td>FITC or AF488</td>
</tr>
<tr>
<td>Orange</td>
<td>535/50 BP</td>
<td>565</td>
<td>572 LP</td>
<td>AF555</td>
</tr>
<tr>
<td>Red</td>
<td>562/40 BP</td>
<td>593</td>
<td>625/40 BP</td>
<td>AF594</td>
</tr>
<tr>
<td>Far red</td>
<td>620/60 BP</td>
<td>660</td>
<td>700/75 BP</td>
<td>AF633 or AF647</td>
</tr>
</tbody>
</table>
Excitation laser  |  Emission           | Fluorescent dyes  
405 nm (Diode)  |  425/50 BP spectral | DAPI           
488 nm (Argon/2)|  525/50 BP spectral | FITC or AF488  
543 nm (HeNe1)  |  560 LP filter      | AF555 or AF594 
633 nm (HeNe2)  |  650 LP filter      | AF633 or AF647 

Table 4.4 Configuration of Olympus confocal microscope
Figure 4.1 The four-color ICC staining of DAPI, CD45, CK, EpCAM on leukocytes, MCF-7 and the enriched cells from TNBC patients.
Figure 4.2 The four-color ICC staining of DAPI, CD45, CK, Vimentin on leukocytes, and the enriched cells from a breast cancer patients
Figure 4.3 The four-color ICC staining of DAPI, N-CAD, CK, Vimentin on the enriched cells from cancer patients
Figure 4.4 The four-color ICC staining of DAPI, CK, EGFR, Vimentin on the enriched cells from cancer patients
Figure 4.5 The four-color ICC staining of DAPI, CK, CD45, HER2 on the enriched cells from cancer patients
Figure 4.6 The three-color ICC staining of DAPI, CK, Smo on the enriched cells from cancer patients
Figure 4.7 The two-color ICC staining of DAPI, CK, Smo on the enriched cells from cancer patients
Figure 4.8 Excitation (dash curve) and emission (solid curve with shadow) spectra of AF555 and AF594, showing relative excitation and emission intensities when excited at 562 nm and detected at 625 nm, respectively.
Figure 4.9 Excitation spectra of selected Alexa Fluor dyes: AF405, AF488, AF555, AF594 and AF647. The gray curve represents the emission spectrum from a typical mercury vapor lamp.
Chapter 5 Combined RNA in situ Hybridization (ISH) and Immunocytochemical Analysis on CTC

5.1 Introduction

Admittedly, the merits of fluorescent ICC staining as described in Chapter 4 are evident. The ICC staining provides intense signals, thus can be applied in most microscopy systems. Meanwhile, as ICC staining targets functional proteins, it can provide the most direct information about the pathological changes of tumor-related cells. However, ICC staining is not always definitive with respect to protein expression levels due to non-specific binding, which is primarily caused by the attraction of primary and secondary antibodies to endogenous Fc receptors (Buchwalow, 2011). To improve the reliability of ICC results, RNA analysis is a complementary staining technology to fluorescent ICC staining in CTC research.

Most RNA analyses, such as quantitative or digital RT-PCR and human genome microarrays, can only analyze RNA isolated from CTCs in aggregated form without
giving specific information about the presence of RNA signal at single cell level. Recently, the single CTC isolation devices developed on the basis of polymer microchip capture and microfluidic magnetic separation have made it possible to capture individual CTCs (Zhao, 2013; Kang, 2012). Some advances in RT-PCR and microarray have been reported to create gene expression profiles for cancer cell lines and CTCs on single cell level (Powell, 2012; Navin, 2011). However, if the tumor-related cells do not fit the definition from EpCAM-based CTC isolation method, which is EpCAM positive, the number of CTCs per ml in the blood samples can exceed 25 for more than one third of cancer patients (Jatana, 2010). Intensive labor and high expense will be required if single CTC isolation and RT-PCR are applied to CTCs of all types in a patient blood specimen. Also, most RNA analysis methods involve cell lysis step, eliminating information about the morphology of cells undergoing analysis, which can be important in identifying and characterizing CTCs for various targets.

To facilitate RNA analysis of CTCs at single cell resolution with direct visualization of targets, fluorescent in situ hybridization (ISH) on RNA is a viable alternative for RT-PCR or microarray analysis. RNA ISH is a visualized technique to fluorescently label the specific nucleic acid sequence on RNA with short anti-sense nucleic acid sequences. Dynamic mRNA transcription and transportation research showed that the quantification
and timeliness of RNA ISH are adequate to track the appearance and location of single mRNA molecule (Femino, 1998). Further, in contrast to the use of antibodies which do not provide targeting information beyond the antibody clones and the approximate antigen location, the probe of RNA ISH is of exactly known sequences. The requirement of sequence length was recently decreased from 50 bp in the initial study to 20bp in the recent studies, which made it possible to label small RNA with RNA ISH (Itzkovitz, 2011).

With these advantages of RNA ISH, several recently published studies on CTCs demonstrated that RNA ISH is able to visualize and quantify EMT markers, Wnt pathway markers and CD45 on tumor-related cells (Yu, 2012 & 2013; Payne, 2012). In these studies, RNA ISH and fluorescent ICC staining were conducted on parallel slides. However, the heterogeneity of CTCs may challenge the validity of parallelism. A combined labelling of RNA ISH and fluorescent ICC staining can possibly be a solution.

In this chapter, we will evaluate several tumor-related markers to label mRNA with RNA ISH on cell lines and various CTCs, including a viral mRNA sequence associated with human papillomavirus (HPV). While Zimmerman et al.’s recent research demonstrated the simultaneous staining of fluorescent ICC and RNA ISH on tissue (Zimmerman, 2013),
as far as we know, we are the first to fluorescently label mRNA and protein on the same CTC. Multi-parameter analysis is performed on the CTCs which are enriched by immunomagnetic depletion of CD45 positive cells from the blood specimens of cancer patients. At the same time, RNA ISH will also be applied on flow cytometry sorting approach to identify and separate cells.

5.2 Materials and Device

The blood source of healthy donor and cancer patient specimens were same as Section 2.3.1. All cell lines were acquired and cultured as Section 2.3.1. The device for CTC enrichment was the QMS device described in Chapter 2. RNA ISH kit applied in this chapter was Quantigene ViewRNA cell assay kit from Panomics. The probes in the RNA ISH were standard probes purchased from Panomics. The microscopy devices, including illuminating equipment, microscope body and filter sets, were the same as introduced in the Section 4.4.2. In additional to Table 4.3, green, orange and far red filters were for fluorochromes of type 4, type 1 and type 6, respectively.

To achieve 6-color staining, the Nuance multi-spectrum image system from CRI Inc. with Model# N-MSI-420-FL was applied. The device has a liquid crystal tunable filter and a
Sony ICX285 CCD camera. The Nuance software from Caliper Life Science was used for spectral deconvolution. To extend spectral options, secondary antibodies and conjugated antibodies with quantum dots were applied. Qdot 585 Donkey Anti-Mouse IgG Conjugate (H+L) and Qdot 655 Donkey Anti-Rabbit IgG Conjugate (H+L), Qdot VIVID from Molecular Probe were used to conjugate mouse-host and rabbit-host primary antibodies, respectively. CD45 Mouse Anti-Human mAb (clone HI30), Qdot 605 Conjugate from Molecular Probe was used to label CD45.

The combined RNA ISH and immunocytochemistry on flow cytometry was carried out by QuantiGene FlowRNA Assay kit (eBioscience). All probes were standard probes purchased from Affymetrix. CD45-AF488 (Clone: HI30) and CD16-AF700 (Clone: 3G8) were purchased from Invitrogen and BioLegend, respectively. BD FACS LSR II, as introduced Section 3.2.3, was used for analysis. The fluorochromes and the corresponding filter/laser sets are listed in Table 5.1.
5.3 Method

5.3.1 RNA *in situ* Hybridization (RNA ISH) Analysis on Slides

20000 magnetically enriched cells from the blood of a cancer patient or healthy donor were used for each RNA ISH slide. For each control slides, 20000 source leukocytes spiked with 5000 cells from a cancer cell line were used. Cell suspensions were spun onto superfrost plus slides and then fixed by 4% paraformaldehyde for 10 min. Subsequently, the cells on the slides were treated with QuantiGene ViewRNA ISH Cell Assay Kit, following the manufacturer’s protocol. Different nucleic acid probes for type 1, type 4 and type 6 conjugations were used to detect the corresponding sequences on mRNAs. The slides were then mounted with ProLong Gold Antifade Reagent with DAPI and sealed with nail balm.

5.3.2 Fluorescent ICC Staining with RNA ISH on Slides.

20000 magnetically enriched cells from the blood of a patient or healthy donor were used in each RNA ISH slide. For each control slide, 20000 source leukocytes spiked with 5000 cells from a cancer cell line were used. After following the same protocol for RNA ISH analysis described above without the protease-incubation and DAPI mounting step, cells
were then blocked with Normal Serum Blocking Solution (NSBS) for 30 min and washed with PBS for 5 min. Subsequently, one or two primary antibodies from different species of hosts (e.g. HER2, EGFR, CK, etc.) were diluted to 5 ug antibody/ml with Normal Antibody Diluent (MP Biomedicals) and applied to the slides. After the staining with primary antibody, the slides were incubated for 30 min in the dark at room temperature and washed three times with 1X Phosphate Buffer Saline containing 0.5% Tween-20 (PBST). Each washing step was approximately 5 min. The secondary antibody (e.g. AF488 Donkey Anti-Rabbit IgG (H+L) (Molecular Probes), AF647 Donkey Anti-Rabbit IgG (H+L), etc.) was diluted to 5 ug antibody/ml with PBST and applied onto the slides. The slides were incubated for 30 min in the dark at room temperature and washed three times for 5 min with PBST. The slides were finally mounted with ProLong Gold Antifade Reagent with DAPI and sealed with nail balm.

5.3.3 Spectral Deconvolution

Spectral deconvolution requires single color and auto-fluorescence control. The single color control for each RNA ISH probe was made with the same protocol in Section 5.3.1 by applying only one RNA probe. For the single color control for fluorescent ICC staining, the same protocol in Section 5.3.2 was followed with only corresponding
antibody added but without any RNA ISH probe. The auto-fluorescence control was made by following the protocol in Section 5.3.2 without any RNA ISH probe and antibody. However, to ensure the identical cell morphology and eliminate the non-specific binding, the auto-fluorescence control still needs pre-amplifier, amplifier and labeling probes in RNA ISH part, and identical wash steps. For all slides involved in spectral deconvolution, the DAPI should be diluted to 30nM prior to use.

Image cubes under four filter sets, which are blue, green, orange and far red in table 4.3, were respectively captured. Each image cube consists of multiple images taken every 10nm in the specific range of spectrum corresponding to the specific filter set. The ranges of blue, green, orange and far red filter sets are 420nm-720nm, 500nm-720nm, 560nm-720nm and 660nm-720nm, respectively. For all slides, including all-color staining set, single color control and auto-fluorescence, images taken from each of these four filters were involved in image cube. The fluorescent intensity curves from all image cubes for all slides were deducted by the fluorescent intensity curves from autofluorescence first. Then the deducted image cubes of single color were used to perform spectral deconvolution with Nuance software.
5.3.4 Immunocytochemistry with RNA ISH for Flow Cytometry

For patient tests, all cells after CD45-based negative depletion from a blood specimen were applied in the experiment tube. For cell line or healthy donor control, one million cell line cells or leukocytes were applied in the control tube.

In addition to specifically targeted reagents, the cell viability indicator, LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, was added to each tube by the amount of 1ul per million cells. Cells were then incubated in dark for 30min, followed by wash step with 1ml PBS. Conjugated antibodies were added into tubes by the amount of 5ul per million cells. Cells were again incubated in dark for 30min, followed by wash step with 1ml PBS. The manufacturer’s protocol of QuantiGene FlowRNA Assay kit was then applied to continue to stain with RNA probes.

For the single color control for RNA ISH, 3e5 cell line cells or leukocytes were used. The procedure of single color control followed manufacturer’s protocol with single probe. The single color control for viability dye and antibodies use Arc and OneComp beads, as introduced in Section 3.3.
5.4 Result

5.4.1 RNA ISH Staining of CTC on Slides

5.4.1.1. RNA ISH on HER markers

RNA ISH was tested targeting β-actin (ACTB), HER2 and EGFR on BT474 and SCC-4 cell lines (Figure 5.1-5.3). Figure 5.1 is BT474 cell line; Figure 5.2 is SCC-4 cell line; Figure 5.3 presents the negatively enriched cells from a metastatic, TNBC patient, stained using the same protocol and probes. ACTB, EGFR and HER2 use type 4 (Green), type 1 (Orange) and type 6 (Far red), respectively. ACTB, a structural protein which mRNA is expressed in most human cells, was used as a control to ensure that the probe binding and amplification structures are working.

In the RNA ISH test with HER2 and EGFR, more orange dots were observed on SCC-4 than BT474, while more white (far red) dots were observed on BT474 than SCC-4. Those results indicate that SCC-4 is more positive than BT474 on EGFR, while BT474 is more positive than SCC-4 on HER2. This observation is consistent with the fluorescent ICC staining on BT474 and SCC-4. In Figure 5.3, the appearance of HER2 positive cells demonstrates the heterogeneity of CTCs.
5.4.1.2. RNA ISH specificity test

After successfully staining with RNA ISH on cell lines and enriched cells from cancer patient blood, the specificity of the RNA ISH was investigated using sense probes. The sense probe is designed with the same amplification and reporting structure but the complementary binding sequence of the RNA ISH probe we applied (anti-sense probe). As expected, the sense probe had zero specific binding capacity toward target mRNA transcript.

Both sense and anti-sense probes on HER2 and EGFR were used in the RNA ISH on the spiking test of BT474 and blood cells with the ratio of 1:4 in cell numbers. In Figure 5.4, (1)-(4) are the slide with DAPI, CK 8,18,19-type 4, EGFR-type 1; (5)-(8) are the slide with DAPI, CK 8,18,19-type 4, sense-EGFR-type 1. (9)-(12) are the slide with DAPI, CK 8,18,19-type 4, HER2-type 6. (13)-(16) are the slide with DAPI, CK 8,18,19-type 4, sense-HER2-type 1. Since BT474 cell line is positive on CK 8, 18 and 19, the mixture of CK 8-type 4, CK 18-type 4 and CK 19-type 4 anti-sense probes was employed as a positive control to ensure the probe binding and proper amplification. The result shows that blood cells do not have any positive signal, while BT474 cells have positive signal
only with anti-sense probes, indicating the sufficient specificity of RNA ISH probes on oncogenes.

5.4.2 Combined RNA ISH and ICC Staining on Slides

5.4.2.1. RNA ISH with ICC Staining – Surface Antigen Target

As is well known, mRNA expression does not necessarily correlate to protein expression. Therefore, an ideal multi-parameter analysis of CTCs that uses RNA ISH and fluorescent ICC staining would verify that protein expression and complementary mRNA transcription on the same cell. Figure 5.5 presents the staining of DAPI, EGFR antibody (ICC, conjugated to AF488), EGFR-type 1 (RNA ISH) and HER2-type 6 (RNA ISH) on a mixture of normal blood cells and BT474 cells (ratio of 1:4). Blood cells show no signal, while BT474 cells are consistently low positive on both EGFR fluorescent ICC staining and EGFR RNA ISH. This result is consistent to previous research (Subik, 2010). Figure 5.6 shows the staining of DAPI, HER2 antibody (ICC, conjugated to AF488), EGFR-type 1 (RNA ISH) and HER2-type 6 (RNA ISH). Here, BT474 cells exhibit different extents of positivity on HER2 due to the heterogeneity of cell growth stage and viability. However, the BT474 cells that are highly positive on HER2 in fluorescent ICC staining
show high positivity on HER2 RNA ISH and vice versa.

5.4.2.2. RNA ISH with ICC Staining - Intracellular Antigen Target.

Figure 5.7 presents the staining of DAPI, anti-CK 8, 18, 19, (ICC, pre-conjugated to AF488), EGFR-type 1 (RNA ISH) and HER2-type 6 (RNA ISH) on BT474 cells. Figure 5.8 is the same combinations of staining applied to the enriched cells from metastatic colon cancer patient. Beyond traditional CTC markers, interest exists on specific pathways related to cancer treatment, such as the Hedgehog pathway. As a cell line control, Figure 5.9 presents the staining of DAPI, CK 8, 18, 19-type 4, Smo-type 1 and Gli-1-type 6 on T47D cells, which is a Hh pathway active cell line. Figure 5.10 is the staining with DAPI, CK 8, 18, 19 (ICC, pre-conjugated to AF488) antibody, Smo-type 1 and Gli-1-type 6, applied to the enriched cells from metastatic breast cancer patient.

5.4.2.3. RNA ISH on Viral Transcripts.

HPV16,18 infection is associated with only basal cells of the stratified epithelium, and is associated with specific types of cervical and head and neck cancer. Of the nine genes related with HPV, genes E6 and E7, and the corresponding proteins, are associated with
anti-apoptotic properties of HPV infection and these gene products are commonly used to signify the presence of HPV. Figures 5.11 and 5.12 are representative, stained images of SCC90 and SCC47 cells mixed with normal blood, respectively. The cells, which are leukocytes spiked with SCC90 or SCC47 cells at a ratio of 4:1 in cell number, were stained with CK8,18,19-AF488 (ICC, pre-conjugated), HPV16 E6/E7-type 1 (RNA ISH) and EGFR-AF647 (ICC). The HPV16 E6,E7-type 1 probe is the 1:1 mixture of HPV16 E6-type 1 and HPV16 E7-type 1. In the HPV RNA ISH channel, the SCC47 cells have fewer dots than SCC90. This fact is consistent with the general ICC staining result indicating that SCC90 is more positive than SCC47 on HPV16. Figure 5.13 is a similarly staining on the enriched cells from head and neck cancer patient that was diagnosed with positive HPV solid tumor. Among these enriched cells shown in Figure 5.13, HPV+, EGFR+, CK+ or – cells are found. As a further control, Figure 5.14 presents a mixture of normal blood spiked with BT474 at a ratio of 4:1 in cell number and stained with the same protocol/reagents as in Figure 5.11-5.13. The BT474 is negative on HPV 16 E6/E7, which indicate the specificity of HPV probes.
5.4.2.4. RNA ISH on mRNA for enzyme marker.

Tumor-upregulated enzymes are not overexpressed as much as the targets discussed above, especially in CTCs. With the 8000-fold amplification in our RNA ISH, a single copy of mRNA transcript of some enzymes can be detected and counted, protein arginine methyltransferase 5 (PRMT5) for example. Leukocytes spiked with MCF-7 at a ratio of 4:1 in cell count were labeled by the combined RNA ISH and ICC staining with the setting of CK8,18,19-AF488 (ICC, pre-conjugated), EGFR-type 1(RNA ISH) and PRMT5-type 6(RNA ISH) (Figure 5.15). MCF-7 cells show positive on CK, EGFR as well as PRMT5, while the leukocytes are negative in all of these markers. A few dots on MCF-7 cells can be observed in the far red channel, representing the low transcription of PRMT5. The same staining setting was also applied to the enriched cells from an esophageal cancer patient blood (Figure 5.16). PRMT5 positive CTCs are found in the staining.
5.4.3 6-color Combined RNA ISH and ICC Staining with Multispectral Imaging System

Nuance multispectral imaging system (Abbr. Nuance), as introduced in Section 4.1.2, can ummix spectral overlapping by spectral deconvolution. Together with quantum dots, this device realizes 6-color combined RNA ISH and ICC staining on the same slide which is crucial for CTC multi-parameter analysis at single cell level. 6-color staining test was carried out by the setting of DAPI, CK8-type 1 (RNA ISH), CK8,18,19-AF488 (ICC, pre-conjugated), CD45-Qdot585 (ICC, pre-conjugated), HER2-Qdot655 (ICC) and HER2-type 6 (RNA ISH) on leukocytes spiked with BT474 at a ratio of 4:1 in cell count (Figure 5.17). In Figure 5.17, BT474 cells are positive in CK8 (RNA ISH), CK8,18,19 (ICC), HER2 (ICC) and HER2 (RNA ISH) and negative on CD45, while leukocytes are positive on CD45 and negative on all tumor-related markers.

5.4.4 Flow Cytometry Analysis on RNA ISH and Immunocytochemistry

5.4.4.1 Flow RNA ISH Test on HER Marker

The initial flow RNA ISH try was carried out on CK8,18,19-type 4, EGFR-type 1 and HER2-type 6 (Figure 5.18). These probes have been proven effective in RNA ISH slide
staining, thus were ideal candidate for the Flow RNA ISH test. This flow RNA ISH was performed on leukocytes spiking with $4 \times 10^5$ MCF-7 cells and $3 \times 10^5$ BT474 cells. Considering most leukocytes are much smaller than tumor cell line, the gate was set on SSC and FSC to tell leukocytes apart. In the following part of Section 5.4.4, leukocytes will also be recognized by this way. In Figure 5.18, comparing with cell line and leukocytes, there are distinct shifts between leukocytes and cell line on CK, HER2 and EGFR channels, which indicate the validity of Flow RNA ISH on cell line. After the successful progress on spiking test, the pre-enrichment and post-enrichment cells from a peritoneal adenocarcinoma patient were tested on the same setting as that in Figure 5.18 (Figure 5.19). In the comparison against pre-enriched cells, at the very right of enriched cells on HER2 marker, there is a little peak corresponding to the HER2+ cells in patient samples which are rare based on the result of fluorescent ICC staining on slides.

**5.4.4.2 Specificity test for flow RNA ISH**

Flow RNA ISH shares the same probes with RNA ISH used for microscope slides, but differs in amplification reagents. Since the binding specificity test of RNA ISH probes towards mRNA transcripts has been tested with sense probes in Section 5.4.1.2, the specificity test of flow RNA ISH should be mainly focused on the non-specific binding of
amplifying reagents and labeling probes. The specificity test applied the setting of CK8,18,19-type 4, EGFR-type 1 and HER2-type 6 on 1e6 leukocytes spiking with 4e5 MCF-7 cells and 3e5 BT474 cells, as well (Figure 5.20). In the negative control set, the same protocol is followed without adding RNA ISH probes. Comparing the median values of the flow results with and without probes, a 10-fold difference can be found.

5.4.4.3 Flow RNA ISH with Combined RNA ISH and Immunocytochemistry

Like RNA ISH staining on the slides, the flow RNA ISH can incorporate immunocytochemical staining. The experiments were performed with leukocytes spiking with 4e5 MCF-7 cells and 3e5 BT474 cells under the setting of CK8,18,19-type 4 (RNA ISH), EGFR-type 1 (RNA ISH), HER2-type 6 (RNA ISH) and CD16-AF700 (ICC, pre-conjugated) (Figure 5.21). The fluorescent intensities of cell line on all RNA ISH channels stay the same as Figure 5.18 and are higher than the signals from leukocytes. Therefore, the RNA ISH signal is not interfered by the prior immunocytochemical staining. For the CD16 channel, two subpopulations (CD16+ and CD16-) exist in the leukocytes, of which CD16+ population is smaller. This result matches the fact reported previously (Sánchez-Torres, 2001). The peak of tumor cell line on CD16 locates between the two peaks of leukocytes, which is regarded as negative but with higher intensity than
the CD16-negative leukocytes due to the higher autofluorescence originated from the larger cell size.

5.4.4.4 Flow RNA ISH on Viral Transcripts

RNA ISH targeting viral transcripts usually requires higher laser voltage due to the low expression level. As a result, additional noise signal may disturb the validity of RNA ISH on viral transcript. Figure 5.22 is the flow result of HPV16 E6E7-type 1 for 1e6 blood cells spiked with 3e5 SCC90 cells. The flow intensity of SCC90 is higher than that of leukocytes, which proves the effectiveness of HPV test on flow RNA ISH.

5.5 Discussion

5.5.1 Combined RNA ISH and Fluorescent ICC Staining on Slides

As briefly stated in the introduction part, though a careful blocking treatment with multiple serums has been conducted for the slide before staining, fluorescent ICC staining is still hassled by the argument of non-specific bindings. Even if non-specific binding is eliminated, the antigens on cell debris which is from cell lysis in the
permeablizing step, can also bind to antibody, introducing the background noise. Meanwhile, the binding capacities between primary antibody and antigen, and between primary antibody and secondary antibody change a lot. Therefore, the quantification of antigen expression may not be easy in fluorescent ICC staining. However, the target of fluorescent ICC staining, protein, can characterize circulating tumor cells in a more direct way than any types of nucleic acid analysis, as the protein is the ultimate functional unit. Moreover, judging by the exposure time and the gain required, the fluorescent ICC staining can emit more intense fluorescent signals than RNA ISH by at least 3-5 folds. The shorter exposure time can accelerate CTC searching on slides.

Since the complementary binding of nucleic acid has a higher specificity than the immune binding between antibody and antigen, the non-specificity of RNA ISH is not as serious as that of fluorescent ICC staining. This argument is also supported by our test on the specificity of RNA ISH.

Due to the high specificity, with proper amplification, RNA ISH is able to track the single transcript on mRNA or microRNA and perform quantitative analysis of gene transcription. The quantitative analysis is precise enough to create a dynamic profile on the synthesis, transportation and degradation of nascent mRNAs after a chemical induction of the
transcription of a certain gene (Femino, 1998). Some later report developed a computational identification procedure with multiple singly labeled probes to count the RNA transcripts automatically (Raj, 2008). Nevertheless, despite the 8000-fold amplification in our RNA ISH probe design, the signal from RNA ISH is still much weaker than fluorescent ICC staining. For our target markers, it is still not confirmed whether the number of transcripts is proportional, or at least positively related, towards the corresponding protein expression and function.

Consequently, in CTC research, both RNA ISH and fluorescent ICC staining possess respective merits. The combination of RNA ISH and fluorescent ICC staining on the same slide can integrate the advantages of both labeling methodologies and allow to choosing a better labeling way for certain markers on CTC multiparameter analysis. Previously, without this technology, researchers use parallel slides to fluorescently visualize the existence of mRNA transcripts and proteins (Yu, 2013). However, the high heterogeneity of CTCs requires all the analysis conducted on the same cell.

Moreover, unlike tumor tissue staining, CTC staining on slides may face the challenge in searching for the specific type of CTCs. In the previous research, the superiority of negative enrichment has been demonstrated, as the negative enrichment can retain
heterogeneous CTCs without the limitation from any single tumor-related marker (Balasubramanian, 2012). However, the enriched cells after negative enrichment may contain a fair number of other cell types to be excluded. Therefore, all RNA ISH staining, which only emits weak fluorescent signals, requires the combination with fluorescent ICC staining on certain typical markers as a searching tool for target cells to analyze in detail with long exposure time. The combined RNA ISH and fluorescent ICC staining methodology on the same cell is thus significant for fluorescent CTC analysis on slide.

5.5.2 Staining Pattern and Atypical Cells

With the combined RNA ISH and fluorescent ICC staining, markers of interest in potential tumor-related cells can be visualized in properly selected method. Two major staining patterns were applied in this report.

The staining pattern of CK (ICC), EGFR (RNA ISH) and HER2 (RNA ISH) is to explore HER family targets. The signaling cascades related to proteins in HER family have important prognostic and predictive significance in a variety of solid tumors and have several targeted therapies that are currently approved or in development. Therefore, the characterization of CTCs for the presence of these drugable targets is of significant clinical interest since CTCs may give the most dynamic snapshot of the state of tumor
progression. For the blood specimens from HER2 positive breast cancer patients, an average of 1517 HER2 positive cells per ml of blood show up in HER2 RNA ISH. According to pathological test, the patients enrolled are not all HER2 positive. However, we found at least one HER2 positive CTC in each patient blood specimen. The appearance of HER2 positive CTCs in pathological HER2 negative patients illuminates the heterogeneity of CTCs.

The staining pattern of CK (ICC), EGFR (ICC) and HPV16 (RNA ISH) is to explore the expression of HPV16 E6/E7 in CTCs from head and neck cancer patients. HPV, which is associated to about 80% oropharyngeal cancer and 40% squamous cell carcinoma, is an important drug and vaccine target. (Marur, 2010). Contributing to the degradation of cell apoptosis inducer p53 and inactivation of tumor suppressor pRB, HPV16 E6 and E7 are the major targets in previous immunofluorescent staining. RNA ISH on HPV16 E6/E7 is applied as a supplement to the pathological test and analysis on individual CTCs. As shown in Figure 5.13, heterogeneous CTCs of CK+, EGFR+, HPV+ and CK-, EGFR+, HPV+ are found in a pathologically diagnosed HPV positive patient. The revelation of different populations of HPV positive tumor-related cells may contribute to more detailed analysis on HPV diagnosis.
In both patterns, CK, stained with fluorescent ICC staining, serves as a primary marker to search for tumor related cells. Part of the benefit of RNA ISH / ICC combined staining is the ability to apply fluorescent ICC staining on common markers to perform a quick search, followed by a detailed analysis on weak or new markers.

5.5.3 Specificity of RNA ISH and Outlying Signals

The specificity test in this research was carried out in three ways: (1) RNA ISH tests with and without probes were performed to detect the non-specific binding between the amplification tree and mRNA transcript; (2) sense and anti-sense probes were applied to test the non-specific binding between probes and mRNA transcripts; (3) probes were tested on cell lines which are positive or negative on the corresponding sequences, that is, in this case, HPV16 E6/E7 probes to SCC90 and BT474 cells. As shown in Section 5.4, the specificity of our RNA ISH can be proved by the outcome of these three tests.

In some images taken from RNA ISH slides, despite the careful optimization on 4% paraformaldehyde fixing time and protease treatment, a few dots, which indicate the positiveness of RNA ISH of a certain sequence on mRNA at a certain location, are found far away from any cells. Taking the staining of HPV16 E6/E7-type 1 on leukocytes
spiked with SCC90 cells as an example, some weak orange outlying dots (HPV RNA ISH) exist far away from SCC90 cells (Figure 5.11). Initially, these weak outlying dots were regarded as a sign of non-specific binding towards apoptotic cells or some other background impurities. Yet in the staining of HPV16 E6/E7-type 1 on leukocytes spiked with BT474 cells which are negative on HPV, the outlying dots do not show up (Figure 5.14). The BT474 test shows that the outlying dots are probably not caused by the non-specific binding. One hypothesis of this phenomenon is the leakage of mRNA fragments. The RNA ISH requires intensive permeabilization to facilitate nucleic acid probes, amplifiers and fluorochromes diffusing across the cell membrane. If the permeabilization of high intensity is conducted, the mRNA, which may be fragmented by the prior formalin fixing step, has a chance to leak out and bind to nucleic acid probes. Evidence supporting this hypothesis is that in the neighborhood of excessively permeablized, lysed or apoptotic cells which are weak on DAPI, the RNA ISH staining dots tend to leak out more. Another hypothesis for outlying staining dots is mRNA sequences in exosomes. The exosomes secreted from some cancer cell lines have been found to contain many tumor-related mRNA sequences by RT-PCR and RNA microarray (Jenjaroenpun, 2013; Chiba, 2012). Therefore, it is possible for exosomes to contain the tumor-related mRNA sequences involved in the RNA ISH staining. A combined staining
of ICC staining with tetraspanins, such as CD9, CD63, and CD91, and RNA ISH is to be
designed to explore the possibility of this hypothesis.

5.5.4 Color Selection in Combined Fluorescent ICC Staining and RNA ISH on Slides

The RNA ISH method applied in this paper creates a signal amplification tree with
branched DNA. This amplification tree can provide 400 binding sites for each labeling
probe and thus reach an 8000-fold amplification for one mRNA target sequence, which
makes it possible to observe every target transcript. However, as stated before, the signals
from RNA ISH are still weaker than fluorescent ICC staining. Image of RNA ISH is
preferred to be captured with non-laser light source, since the high voltage, which is
required to visualize weak RNA ISH signals on laser, may bleach the fluorochrome
bound to RNA ISH probe. In our 4-color staining setting with mercury bulb as exciting
light source, the excitation peaks of the green (AF488 on ICC or type 4 on RNA ISH) or
orange (AF555 on ICC or type 1 on RNA ISH) channel can catch the peaks of the
emission curve of mercury bulb, which make the green and orange channels more
applicable to markers with weak signals (Wu, 2013). Accordingly, in this report, RNA
ISH on weakly expressed markers should be designed on the orange and green channels,
such as EGFR-type 1, HPV E6/E7-type 1, and ACTB-type 4. Meanwhile, fluorescent
ICC staining or RNA ISH with high overexpression level may use the far red channel, for example, EGFR-AF647 and Her2-type 6. The only exception against this rule in this report is CK-AF488 ICC staining. Since most CTCs are CK positive, CK in this case is designed for a quick and rough pick of CTCs on the slide. To expedite CTC initial recognition, staining CK by ICC on for green channel can shorten the exposure period required by CK scanning.

5.5.5 Color Selection in 6-color Staining on Slides

The emission spectra of the 4-color staining setting of AF405/DAPI, AF488, AF555/AF594 and AF647 occupy the entire spectrum which is detectable by camera sensor (Figure 4.9). If the staining pattern needs to be extended to 6 colors, some labeling probes should share emission spectrum. Despite distinct excitation spectra, the similar emission spectra can cause serious spectrum overlapping. Nuance camera can reverse the spectrum overlapping by spectral deconvolution with the emission and excitation curves of autofluorescence and every single color.
Theoretically, once two labeling probes don’t have identical emission curves, their signal can be distinguished by spectral deconvolution. However, several factors can influence the validity of spectral deconvolution.

Figure 5.23 is the single color curves for all fluorochromes used in spectral deconvolution for the staining shown in Figure 5.17. The intensities of autofluorescence in orange filter and far red filter can reach 10-20% of intensities of the highest emission peaks of respective filters. For the low emission peak under a certain filter, HER2-Qdot655 in far red filter for example, the autofluorescence can be as intense as the fluorochrome emission. Meanwhile, considering autofluorescence emitted by cover glass, cell cytoplasm, surrounding lighting, etc., in our control test, it can vary up to 50% within parallel slides. Accounted for the large proportion of autofluorescence in the emission from fluorochromes, the variation of autofluorescence can miscalculate the actual single color spectral curve for spectral deconvolution.

The underlying presumption of spectral deconvolution is that the excitations and emissions of fluorochromes are not mutually influenced. However, the cross-correlation of different fluorochromes in immunochemical staining can slightly distort the emission curve of individual fluorochrome (Bacia, 2006). For two markers of similar emission
intensities at respective channel, such as DAPI and CK-AF488 in Figure 5.23, this distort won’t influence much on spectral deconvolution. However, if the emission intensity of one marker is much higher than another, for example a weakly positive marker in RNA ISH staining vs a highly positive marker in positive ICC staining, a slight distortion in the emission curve of the markers with higher emission can result in a significant miscalculation for the intensity of the markers with lower emission in spectral deconvolution. This is because some fluorochromes have a broad range of excitation wavelength, thus can be excited and emit in neighbor channel which is next to its main excitation and emission channel. HER2-Qdot655 in Figure 5.23, for example, can also be excited on green and orange filters and has about ~30% and ~50% emission intensity of that in blue filter. If the Qdot655 emission from orange filter exceeds the emission of CK8-type1 which is majorly excited and emits in orange channel (Although it does not happen in Figure 5.23), the spectrum distortion of Qdot655 can significantly influence the deconvolution of CK8-type1.

Therefore, the smaller difference on emission intensities of all markers is key criterion to arrange colors for 6-color staining. Among all the fluorochromes applied in the 6 color staining, the quantum dots are the brightest, followed by Alexa Flour, while the RNA ISH staining is the weakest. As discussed in Section 4.7.3, green and orange channels, which
can excite fluorochromes with more intense light source due to the emission spectrum of
mercury bulb, are more suitable for weak markers. However, most quantum dots can be
partially excited in the green channel. As a result, the signal of RNA ISH staining in
green channel is usually much lower than the emission of quantum dots in green channel,
hence are influenced by spectrum distortion of quantum dots as discussed above.
Therefore, orange channel is optimal for RNA ISH, while far red channel should be fine
for RNA ISH, considering some type 6 RNA ISH were successfully stained in the
combined RNA ISH and ICC staining. Moreover, to match the brightness of RNA ISH,
secondary antibodies of Qdot should be diluted. In our 6-color setting, Qdot 585 and
Qdot 655 were diluted to 2nM and 1nM, while all secondary antibodies with Alexa Fluor
were diluted to 5nM.

5.5.6 Color Selection in Combined Fluorescent ICC Staining and RNA ISH on Flow
Cytometry

Unlike the spectral deconvolution of Nuance, the compensation of flow cytometry is
based on emission intensity values at some certain wavelength but not the curves
covering the whole spectrum. Therefore, the compensation in flow cytometry is more
likely to be influenced by spectral distortion and high autofluorescence discussed in
Section 5.5.5. As a result, the color choice of combined ICC staining and RNA ISH on flow cytometry should be very careful. As listed in Table 3.2, 8 color channels are available except for the viability indicators. The RNA ISH probes for flow cytometry are designed as type 1, type 4 and type 6, as listed in Table 5.1. The genes weakly transcribed should use the type 1 channel, while the genes strongly transcribed should use the type 4 and type 6 channels. The reason is type 4 channel has higher background noise and autofluorescence due to the shorter wavelength in excitation light, while, since the long wavelength in excitation light, the fluorochrome of type 6 may require a higher excitation luminous intensity to emit the equivalent intensity of fluorescence.

The immunocytochemistry staining in flow cytometry can be 3-5 folds brighter than flow RNA ISH. Therefore, the fluorochromes of ICC staining, especially for those markers highly expressed, should be spectrally far away from RNA ISH probes. Those lowly expressed markers can be a little closer spectrally, CD16 for example.

5.6 Conclusion

As far as we know, this is the first report on a staining methodology of RNA ISH and fluorescent ICC staining on the same CTCs. The combined staining of RNA ISH and
fluorescent ICC staining enables us to visualize the characterization of a single CTC on both protein and RNA levels. To achieve high throughput performance, flow cytometry was successfully applied in the combined staining of RNA ISH and fluorescent ICC on cell line and CTCs. Meanwhile, this is also the first report to apply multi-spectral imaging technique on RNA ISH staining with 6-color labeling on the same cell.

With these methodologies, several markers of special attention by prevailing researches were used in CTC labeling, including HER family markers, HPV, PRMT5, etc. As a result of heterogeneity, some CTCs are against pathologic judgment on HER2 and HPV. Therefore, the test on individual CTCs may supplement the current prevalent tissue-based pathologic diagnosis and extend our vision on these markers.
5.7 Reference


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Table 5.1 Configuration of flow cytometer for RNA ISH / immunostaining
Figure 5.1 RNA ISH staining of β-actin (ACTB), HER2 and EGFR on BT474 cell line
Figure 5.2 RNA ISH staining of β-actin (ACTB), HER2 and EGFR on SCC-4 cell line
Figure 5.3 RNA ISH staining of β-actin (ACTB), HER2 and EGFR on the enriched cells from the blood specimen of breast cancer patient
Figure 5.4 Sense and anti-sense probes test of HER2 and EGFR on leukocytes spiked with BT474
Figure 5.5 Combined RNA ISH and ICC staining with EGFR (ICC), EGFR (RNA ISH) and HER2 (RNA ISH) on leukocytes spiked with BT474
Figure 5.6 Combined RNA ISH and ICC staining with HER2 (ICC), EGFR (RNA ISH) and HER2 (RNA ISH) on leukocytes spiked with BT474
Figure 5.7 Combined RNA ISH and ICC staining with CK (ICC), EGFR (RNA ISH) and HER2 (RNA ISH) on BT474
Figure 5.8 Combined RNA ISH and ICC staining with CK (ICC), EGFR (RNA ISH) and HER2 (RNA ISH) on the enriched cells from metastatic colon cancer patient.
Figure 5.9 RNA ISH staining of CK 8,18,19, Gli1 and Smo on T47D cell line
Figure 5.10 Combined RNA ISH and ICC staining with CK (ICC), Gli1 (RNA ISH) and Smo (RNA ISH) on the enriched cells from metastatic breast cancer patient
Figure 5.11 Combined RNA ISH and ICC staining with CK (ICC), HPV 16 E6/E7 (RNA ISH) and EGFR (ICC) on leukocytes spiked with SCC90
Figure 5.12 Combined RNA ISH and ICC staining with CK (ICC), HPV 16 E6/E7 (RNA ISH) and EGFR (ICC) on leukocytes spiked with SCC47
Figure 5.13 Combined RNA ISH and ICC staining with CK (ICC), HPV 16 E6/E7 (RNA ISH) and EGFR (ICC) on the enriched cells from head and neck cancer patient
Figure 5.14 Combined RNA ISH and ICC staining with CK (ICC), HPV 16 E6/E7 (RNA ISH) and EGFR (ICC) on leukocytes spiked with BT474.
Figure 5.15 Combined RNA ISH and ICC staining with CK (ICC), EGFR (RNA ISH) and PRMT5 (RNA ISH) on leukocytes spiked with MCF-7
Figure 5.16 Combined RNA ISH and ICC staining with CK (ICC), EGFR (RNA ISH) and PRMT5 (RNA ISH) on the enriched cells from esophageal cancer patient.
Figure 5.17 The images after spectral deconvolution of combined RNA ISH and ICC staining with CK8 (RNA ISH), CK (ICC), CD45 (ICC with QD), HER2 (ICC with QD) and HER2 (RNA ISH) on leukocytes spiked with BT474
Figure 5.18 Flow plots of flow RNA ISH with CK8,18,19, EGFR and HER2 on leukocytes spiked with BT474 and MCF-7
Figure 5.19 Flow plots of flow RNA ISH with CK8,18,19, EGFR and HER2 on the enriched cells from peritoneal adenocarcinoma patient.
Figure 5.20 Flow plots of the specificity test of flow RNA ISH with or without CK8,18,19, EGFR and HER2 probes on leukocytes spiked with BT474 and MCF-7
Figure 5.21 Flow plots of the combined flow RNA ISH and immunochemical staining with CK8, 18, 19 (RNA ISH), EGFR (RNA ISH), HER2 (RNA ISH) and CD16 (Immunochemistry) on leukocytes spiked with BT474 and MCF-7.
Figure 5.22 Flow plots of flow RNA ISH with HPV16 E6/E7 on leukocytes spiked with SCC90
Figure 5.23 Single color curves for all fluorochromes used in spectral deconvolution for the staining shown in Figure 5.17 (the AF curve is for autofluorescence in this figure)
Chapter 6 DNA Fluorescent in situ Hybridization (FISH) on CTCs

6.1 Introduction

DNA fluorescent in situ hybridization (FISH) is a staining method to fluorescently label the specific DNA sequence with an anti-sense DNA probe which is conjugated to fluorescent molecules. Comparing with RNA ISH and ICC staining, DNA FISH provides a sight from the number of gene duplicates.

In a typical DNA FISH test, nucleic acid probe for a specific sequence is firstly bound to fluorophore or the protein to connect fluorophore, and nucleic acid sequence. Then the nucleic acid probe with fluorophore and the DNA in cell are both denatured in water bath over 80 °C. After that, the specific sequence on DNA and the anti-sense DNA probe bind together in 3-hour or overnight incubation in 37 °C. Figure 6.1 (Speicher, 2005) presents the flow chart of indirect and direct DNA FISH. In Figure 6.1, (a) is the basic elements of FISH, a DNA probe and a target sequence. (b) presents two common labeling strategies with the DNA probe: indirect labeling (left panel) and direct labeling (right panel). For
the indirect labeling, probes are labeled with the modified nucleotides that contain
haptens, whereas the direct labeling uses nucleotides that have been directly bound to
fluorophores. (c) is the denaturation step of the labeled probe and the target DNA. (d)
presents the step to mix the denatured probe and the target sequence to allow the
annealing of the complementary DNA sequences. In (e), if the probe is labeled indirectly,
an extra step is required for visualizing the nonfluorescent haptens that are used in an
enzymatic or immunological detection system. In the indirect DNA FISH, the nucleic
acid probe is usually bound to hapten and then visualized by immunoassay, while the
probe is directly bound to fluorochromes in the direct DNA FISH.

Superior to RNA ISH and ICC staining, DNA FISH can be precisely quantified by
comparing the numbers of chromosome and target genes (Bofin, 2004). For rare CTCs,
DNA FISH can advance the analysis beyond positive-or-negative judgment and provide a
numerical parameter. Therefore, the precise quantification of individual cells can greatly
promote the correlation between patient condition and CTCs.

DNA FISH has been widely used in the detection of HER2/Neu status. Since the HER2
gene can be excessively synthesized, even if the primary tumor is HER2 negative,
HER2/CEP-7 dual color DNA FISH on CTCs have been approved as a good way to
evaluate the status of tumor progression and to determine the treatment plan (Meng, 2004).

Meanwhile, early research showed that the number of the gene copies of EGFR from EGFR DNA FISH is significantly correlated \((r \approx 0.4, P<0.001)\) to the protein expression from immunohistochemical staining in lung cancer and is relevant to cancer prognosis (Hirsch, 2003). However, in terms of EGFR, a summarizing review also shows immunohistochemical staining, PCR and FISH have a variation in the result of cancer study (Gupta, 2009). As a result, the determination of a standard method is required in the future research. And EGFR DNA FISH is still a competing method to evaluate the tumor progression.

In this chapter, the EGFR and HER2 FISH will be applied onto cell lines and the enriched cells from patient blood specimens. Cells with multiple HER2 or EGFR genes on chromosome will be reported and compared with normal hematopoietic nucleated cells. Clayton Deighan partially participated in the research reported in this Chapter.
6.2 FISH probes and microscopy devices

The FISH probes used in our research were PATHVYSION HER-2 DNA Probe and Vysis LSI EGFR SpectrumOrange / CEP-7 SpectrumGreen Probes from Abbott. Both probes are dual color FISH probes of HER2/CEP-17 and EGFR/CEP-7 with SpectrumOrange / SpectrumGreen. CEP-7 and CEP-17 are chromosome enumeration probe 7 and 17, which are binding to alpha satellite of the centromeric region of human chromosome 7 and 17. These two probes were employed to count the numbers of chromosome 7 and 17 on which EGFR and HER2 gene locate.

The SpectrumGreen can fit the green filter set in Table 4.3, while the SpectrumOrange needs a new filter set of excitation 545/25BP, dichroic 565LP and emission 605/70BP. DAPI in DNA FISH test may still use the blue filter set to observe.

6.3 Method

More than 15 cancer patient blood specimens were processed with the QMS enrichment and then HER2/CEP-7 DNA FISH. The primary tumors of these patients were either head and neck tumor or metastatic breast tumor. The total number of HER2 positive cells on
each slide has been counted to calculate the number of HER2 positive cells per ml blood in each patient blood specimen.

The DNA FISH started with the enriched cell population after the negative depletion. The commercial FISH probes were designed for tissue staining. By optimizing the procedure as follows, we were able to apply them on our enriched cells.

1. 20,000 cells from patient blood or healthy donor, or cell line cells are cytospun onto superfrost plus slides.

2. Pretreat the slides with Saline-sodium Citrate (SSC) buffer for 2min at room temperature. The slides with cells are then washed by 1X PBS.

3. Treat the slides with 2X SSC in 70-75 °C for 15min.

4. Rinse the slides with 10mM HCl and digest nucleic enzyme with 6ul/ml pepsin solution in 10mM HCl for 15min in 37°C

5. Immerse the slides in 70% formamide in 2X SSC at 70-75 °C for 5 min to denature the nucleic acid.

6. Dehydrate the slides in 70%, 80% and 100% ice cold ethanol for 1 min for each and air dry the slides.
7. 10ul LSI HER-2/neu SO/CEP 17 SG probe from PATHVYSION HER-2 DNA Probe Kit or 10ul Vysis LSI EGFR SpectrumOrange / CEP7 SpectrumGreen Probe are mounted onto the slides to hybridize the nucleic acid with probes.

8. Cover the slides by coverslip and seal with nail balm. Incubate the slide in 37°C for 15-18 hours.

9. The coverslip is removed and the slide is washed with 0.4x SSC/0.3% NP-40 in 70°C for 2min and subsequently 2x SSC/0.1% NP-40 under room temperature for 1min.

10. Mount the slides with ProLong Gold Antifade Reagent with DAPI and seal with nail balm again.

6.4 Result

The CEP-7 and CEP-17 were labeled by SpectrumGreen, while the target genes were labeled by SpectrumOrange. There should be two green dots corresponding to two chromosome 7’s or two chromosome 17’s. However, cell lines, CTCs and normal cells can all possibly undergo mutation or mitosis. Therefore, more than two green dots can possibly exist in a cell. Theoretically, each chromosome includes up to 2 HER2 or EGFR genes in normal cells. Yet the ratio of chromosome and target gene is usually less than 2,
due to the frequent gene deletion. Any ratio higher or equal to 2 will be considered as gene amplification. “Ratio” in the following of this chapter will be considered as the ratio of target gene duplicates over chromosome enumeration probes.

Figure 6.2 is the SCC-4 cell line control for the EGFR DNA FISH staining. The SCC-4 cells with 2, 3 and 4 chromosome 7’s have 4, 7 and 9 EGFR genes, respectively. The corresponding ratios are 2, 2.3 and 2.25, which indicate the amplification of EGFR genes in SCC-4 cells. The EGFR DNA FISH was subsequently applied to the enriched cells from metastatic breast cancer patient (Figure 6.3). The cells with ratios of 1.5 and 2 were found in the same microscopy field of view in Figure 6.3. The HER2 DNA FISH was carried out on the enriched cells from a colorectal cancer patient who is pathological HER2 positive (Figure 6.4). From this HER2 DNA FISH, one cell with a ratio of 1.5 is determined as HER2 negative, while the other one with a ratio of 3 is determined as HER2 positive.

6.5 Discussion

In this chapter, DNA FISH protocol has been developed on HER2 and EGFR. The cell line control on EGFR verifies the reliability of our methodology. The cells with amplified
HER2 and EGFR genes from cancer patient blood specimens reveal the probability of HER family marker amplification in CTCs, which indirectly supports the discovery of HER2 and EGFR circulating free DNA reported previously (Page, 2011; Kim, 2013).

Compared to with RNA ISH and fluorescent ICC staining, the DNA FISH is quantitative and has a reasonable level of internal control. For example, when counting the dots created by the DNA FISH probes, any dots far from DAPI staining should not be considered. For the cells from patients who are taking or just finished chemotherapies with high dose, the nucleus morphology viewing under DAPI is probably jagged due to the partial lysis of nucleus. In this situation, we should count the dots close to DAPI staining (not necessary on the DAPI staining). On the contrary, if the cell nucleus looks normal, any dots beyond the DAPI staining should be neglected.

Like type 1 in RNA ISH and AF555 in fluorescent ICC staining, fitting in the orange channel, the SpectrumOrange can emit more intensively than the fluorochromes using other channels. As a result, the SpectrumGreen can be influenced by the spectral leaking from SpectrumOrange. In the “merge” images of Figure 6.2, some dim green dots for CEP7 and CEP17 can completely overlap the bright orange dots for EGFR and HER2.
These green dots are probably formed by the spectral leaking from SpectrumOrange, thus should not count for centromere.

DNA FISH provides information with respect to the DNA content of the cell, which is not covered by fluorescent ICC staining and RNA ISH staining. Although the DNA FISH cannot provide the direct information on the functional or structural change of CTCs (protein is the ultimate functional unit), the highly quantitation of DNA FISH can provide very precise information of gene amplification, which is useful in CTC quantitative characterization and has been linked to several specific types of cancer. With DNA FISH, RNA ISH and fluorescent ICC staining, an integrated methodology covering protein, RNA and DNA levels to analyze CTCs is established. As an example in this document, HER family markers (EGFR, HER2) were tested in all DNA FISH, RNA ISH and fluorescent ICC staining, which have very diverse results. The disparity is possibly contributed by the disaccord of gene amplification, overtranscription and overexpression, for the fact that abundance in gene duplicates does not mean high transcription and expression. Meanwhile, the disparity can also be contributed by the high heterogeneity of CTCs.
To quantify the CTC heterogeneity, a combined staining of DNA FISH and fluorescent ICC staining is under development. Some early literature reported the methodologies to stain DNA FISH and ICC simultaneously (Mialhe, 1996; Speel, 1994). However, the target markers in these studies were highly amplified or expressed. For the tumor related markers in CTC research, the target gene sequence or protein are usually very weakly amplified or expressed. Further, the high temperature for DNA melting can denature protein, while the fixing step in fluorescent ICC staining may fragment DNA. A compromise on DNA melting temperature and the formalin concentration in protein fixing needs to be tested to develop combined DNA FISH and ICC protocol for CTC study.
6.6 Reference


Figure 6.1 The flow chart of typical indirect and direct DNA FISH
Figure 6.2 SCC-4 cell line control for the EGFR DNA FISH staining
Figure 6.3 EGFR DNA FISH on the enriched cells from metastatic breast cancer patient
Figure 6.4 HER2 DNA FISH on the enriched cells from a pathological HER2+ colorectal cancer patient
Chapter 7 Exploring Potential Tumor-related Markers for CTC

Research

7.1 Introduction

Some markers for CTC analysis have been reported and tested, as described in Section 4.3. However, a typical CTC has, presumably, undergone loss of cellular adhesion, increased motility and invasiveness, and finally enters and survives in the circulatory system (Gupta, 2009). During the long process from original tumor to circulatory system, many markers may be involved in the procedure of cell morphology change and migration. To explore new tumor-related markers for CTC analysis, RNA microarray (i.e. Nanostring nCounter array) can give a direction in the search for CTC markers. Despite the fact that the result of microarray analysis is an average of 1000 or more cells but not at single cell resolution, the ability in high throughput screening can speed up the exploration of CTC markers.
In this chapter, some microarray products will be compared and applied on CTCs. High throughput screening will be performed on cell line and the enriched cells from patient blood specimens. By microarray analysis, some potential markers will be found closely related to patient prognosis.

7.2 mRNA Microarray Analysis

Generally, there are two approaches to analyze mRNA with microarray: one approach is to reversely transcribe mRNA to cDNA, amplify cDNA with PCR and then label the gene for detection; the other approach is to use mRNA directly, label the mRNA sequences with probes and amplify the signal from probes. In the first approach, empirically, the reverse transcription step requires 10-50ng of total RNA, which is usually not easy to reach in CTC study, to get cDNA of adequate quality and quantity. Meanwhile, the activity of reverse transcriptase is sensitive to the salt and phenol in the RNA lysate after RNA isolation. The second approach is more sensitive due to the direct binding. But the enhanced variation in the high signal amplification can influence the precision of quantification.
Evaluating by eligibility and accessibility, we picked three prevailing microarray products to perform our CTC analysis: 3' IVT Expression Analysis with Human Genome U133 Plus 2.0 Array (Affymetrix), nCounter Single Cell Expression Assay (Nanostring) and QuantiGene 2.0 Multiplex Assay (Panomics).

7.2.1 3' IVT Expression Analysis

In 3' IVT Expression Analysis with Human Genome U133 Plus 2.0 Array, the mRNA is initially reversely transcribed to cDNA with T7-Oligo(dT) primer. dsDNA, which is synthesized by cDNA, transcribes to aRNA (amplified RNA) with biotinylated ribonucleotide analogs. The aRNA with biotinylated ribonucleotide is then fragmented and complementarily bound to genome array. The probes on the genome array bound with biotinylated aRNA fragment can be fluorescently detected by treating with streptavidin- R-Phycoerythrin (PE).

With Human Genome U133 Plus 2.0 Array, 3' IVT Expression Analysis can reveal the tumor cell line expression change at drug stimulation in different environments (Zschenker, 2012; Tabuchi, 2012). 3' IVT Expression Analysis is also applicable for the RNA extracted from patient tumor tissue to evaluate tumor progression in clinical study.
(Schmitz, 2012). Using the Human Genome U133 Plus 2.0 Array, 3' IVT Expression Analysis can test the expression of over 47000 genes on ~50ug total RNA. Yet for rare CTCs, the amount of total RNA is below 10ng, which is below the minimum input requirement of 3' IVT Expression Analysis.

### 7.2.2 QuantiGene Plex 2.0 Assay

QuantiGene Plex 2.0 Assay is derived from the Quantigene ViewRNA technique applied in RNA ISH. Target mRNA sequences are labeled and immobilized onto Luminex magnetic beads via a nucleic acid molecule called Capture Extender. Then target sequences are recognized by Label Extender via which the amplification structure can label the target sequence. The amplification structure for QuantiGene Plex 2.0 Assay is very similar to RNA ISH. Pre-amplifier, amplifier and final labeling probe can construct a tree structure to enhance the signals by 2400 folds, with which the Luminex machine can detect every single RNA transcript on PE channel. With the different combinations of Luminex beads and PE, 80 different colors can be recognized by Luminex machine.

26-plex QuantiGene 2.0 Assay has been applied onto formalin-fixed and paraffin-embedded tumor tissues, in which the data cluster can clearly separate two kinds
of tumor tissues (Hall, 2011). In Dr. Timmers lab, one of our collaborative labs in the Ohio State University Medical Center, the QuantiGene 2.0 Assay was performed on breast tumor tissues. The procedure of QuantiGene 2.0 Assay for CTCs is still under developing in our lab.

7.2.3 nCounter Expression Assay

Similar to QuantiGene 2.0 Assay, nCounter Expression Assay uses the biotinylated capture probes, part of which are sense nucleotide sequences of the target genes, to immobilize mRNA on streptavidin-coated cartridges. The assay labels the target sequences on mRNA by labeling probes. The labeling probe, which carries a barcode marker, can be read after stretching on the surface of cartridge well in electrophoresis. And each barcode markers can be individually identified with the sequence order of fluorescent molecules. With 509 different markers tested simultaneously, nCounter Expression Assay can test till 0.1fM RNA with an R-square of 0.9988; meanwhile, the assay can cover RNA concentrations of 6 orders of magnitude with an R-square of 0.9999. (Geiss, 2008)
Previously, different types of tumor tissues applied nCounter Expression Assay to discover the similarity of gene signatures between the basal-like and luminal-like tumors. (Prat, 2013). In some research, successful usage of nanostring on CTCs from pancreatic cancer patients is reported (Sergeant, 2012). Due to the previous success, nCounter Expression Assay is chosen in our initial test of CTC on microassay in seek of potential tumor-related markers.

7.3 Material and Method

All three assays described in Section 7.2 were currently applied in our CTC research. Most enriched cells from patient specimens were analyzed by nCounter Expression Analysis. 22 patient specimens, of which 18 were metastatic breast cancer patients and 4 were head and neck cancer patients, were applied onto nCounter Expression system.

For each patient specimen, total RNA was isolated from all negatively magnetic enriched cells stored in RNAlater for up to 2 years. The RNA isolation was performed with RNA/DNA Purification Micro Kit (Norgen Biotek) by following manufacturer’s protocol. The concentration of RNA and RNA integrity number (RIN) were determined by Agilent
RNA 6000 Pico Kit on Agilent bioanalyzer. All RNA lysates were diluted to the same concentration determined by the specimen with least RNA amount.

To increase the signal intensity in advance, cDNA was synthesized and duplicated through RT-PCR with the pooled primers shown in Table 7.1. Determined by preliminary experiment run with cell lines, 30 cycles were run in PCR step. The nanostring cartridge with 46 gene probes which include 5 housekeep genes was applied on these 22 patient samples. The data was manually processed nSolver Analysis (Nanostring), in which patient specimens with normalization flags were not considered for further data analysis. The clustering was performed by Cluster 3.0 (Stanford University) with centroid linkage, while the heatmap was created by TreeView (Alok Saldanha).

7.4 Result

The RNA amount measured by bioanalyzer from the enriched patient cells is shown in Table 7.2. In Table 7.2, 08140 is the serial number of clinical trial in the Ohio State University for metastatic breast cancer research from which about 200 patient blood specimens were acquired; WR is the blood specimens of suspected cases of breast cancer from Walter Reed Army Medical Center; Head and Neck (HN) indicates the blood
specimens from the patients with head and neck tumor. These are consistent with the blood source introduced in Section 2.3.1.

In the initial test, part of the breast cancer specimens and all 4 head and neck cancer specimens were tested. The clustering after the normalization based on both genes and patient specimens can clearly show two separate clusters on breast cancer patients and head and neck cancer patients (Figure 7.1). Figure 7.2 shows the result of nCounter Expression Assay of all patient samples and MCF-7. The heatmap was only normalized in gene direction but not patient sample direction. In the clustering in Figure 7.2, MCF-7 is far away from any patient samples.

7.5 Discussion

Due to the low minimum detectable threshold, nCounter Expression Assay can analyze rare CTCs very well. As listed in Table 7.2, the RNA concentration of the enriched cells from patient samples can be lower than 10pg/ul. The result of the clustering of the patient samples shows the capability of nCounter Assay to distinguish CTCs from two distinct cancer types.
The RNA amount per cell varies in a very broad range. Part of the reason is the error in cell counting in hemocytometer and cell heterogeneity. Meanwhile, the agilent bioanalyzer can introduce some errors when processing RNA with low RIN and concentration. However, to eliminate the error introduced by the RNA input difference, the assay uses housekeeping genes to normalize the output, assuming the housekeeping genes have the same expression level under the same overall expression activity. To further eliminate the error introduced by the probe difference in each well, positive controls of External RNA Controls Consortium at 128 fM, 32 fM, 8 fM, 2 fM, 0.5 fM, and 0.125 fM are used to normalize. In this way, most errors raised by input and device difference can be eliminated.

To analyze the variance and similarity of patient samples by clustering, normalization on heatmap should be in patient sample direction to offset the similarity at a certain gene, as shown in Figure 7.1. In the quest for potential tumor-related markers, however, normalization on heat map should be in gene direction, since all patient samples can be positive on a closely tumor-related sequence, as shown in Figure 7.2. Vimentin (VIM in figures), a typical mesenchymal marker, for example, is highly positive on all patient samples under gene direction normalization (Figure 7.2) and variable on the patient samples in Figure 7.1. Vimentin in Figure 7.2 indicates its close relation to tumor
occurrence; however, comparing patient-wise, the expression of vimentin still differs a lot in the Figure 7.1.

Aside from 5 housekeeping genes, 31 potential tumor-related genes were chosen. It is labor-intensive and costly to stain all the corresponding RNA transcripts or proteins with RNA ISH or fluorescent ICC staining on CTCs. The result from nCounter Expression Assay can abridge our target lists and highlight some tumor-related markers. Besides some frequently reported markers, such as CD24, CD44, KRT, CDH, etc., BMI1, ERBB2IP, TGFB1, SERPIN1 and CXCR4 are coming into our future research scope.
7.6 Reference


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Table 7.1 Primer sets for Nanostring nCounter Expression test
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<th>Sample #</th>
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Table 7.2 Patient info and RNA input in Nanostring nCounter Expression test
Figure 7.1 Heatmap and centroid linkage of the result of Nanostring nCounter Single Cell Expression Array for the enriched cells from 5 TNBC patients and 4 HN patients on 41 potential tumor-related genes. This heatmap is normalized on both gene and patient specimen directions.
Figure 7.2 Heatmap and centroid linkage of the result of Nanostring nCounter Single Cell Expression Array for the enriched cells from all tested patients and MCF-7 on 41 potential tumor-related genes. This heatmap is normalized only on gene direction.
Chapter 8 Conclusion and Future Direction

8.1 CTC Negative Magnetic Separation

Due to the rarity of CTCs, the CTC separation requires both accuracy and high-throughput processing. The QMS device can process up to 100 million nucleated cells in a single separation step, which correspond to approximately 15-20ml patient blood. The ability of high-throughput processing brings the possibility of analyzing heterogeneous CTCs. Meanwhile, as described in Chapter 2, the commercialized EpCAM-based positive-selection devices for CTC have been criticized by omitting EpCAM-negative tumor related cells. The CD45–based negative magnetic separation can enrich heterogeneous CTCs positive in other tumor related markers. Moreover, the magnetic or fluorescent labeling applied in the CTC sorting process may cause steric hindrance for probe binding in the subsequent analysis, thus limiting the selection of targets and analyzing methodologies. However, the CD45-based CTC magnetic separation can provide labeling-free CTC for downstream analysis, which is the basis for the multi-parameter analysis in this document.
With more than 200 patient specimens processed, an average of 99.72% nucleated cells was removed in the negative enrichment with QMS. An average of 99.998% blood cells was removed in the whole CTC separation procedure. The high removal percentage, combining with the relative small variance of total / nucleated cell $\log_{10}$ depletion, shows a stable effectiveness of the negative magnetic sorting with QMS device.

The future magnetic CTC separation research may focus on three aspects: 1. microfluidic CTC chip, 2. CTC enrichment with multiple labeling, 3. assay automation.

Despite the high removal percentage in the magnetic enrichment, a certain number of cells after enrichment are still CD45 positive. The negative enrichment can remove most lymphocytes which are high positive on CD45, but may retain part of granulocytes and monocytes which are weakly positive on CD45. Therefore, a more precise separation device should be applied into CTC separation. CTC chips have been developed to isolate CTC after CD45-labeling (Nagrath, 2007). To get a higher purity of CTCs, single cell microfluidic chip with controllable magnets were developed to entirely separate magnetic labeled cells (Chen, 2013). One of future research direction on CTC separation is to apply this controllable microfluidic chip to CTC separation. Two major challenges for this application are the cell loss in tubing and chips, and low throughput capacity.
Based on the high precision and resolution of CTC chip, CTC purification with multiple labeling can possibly become the next generation of CTC separation methodologies. CTC chips based on dual labeling of CD45 and CD66b have been employed on patient blood specimen analysis (Karabacak, 2014). In the future study, similar markers can be labeled in the same CTC separation to acquire heterogeneous CTCs. For example, CK, EpCAM and E-CAD can be used to positively magnetically label CTCs in a single separation. Meanwhile, multiple controllable CTC chips can be used in series pattern to categorize nucleated cells. Nucleated cells can be firstly labeled with CD45-magnetic beads and processed through CTC chip. Then the cells without magnetic labeling can be labeled with magnetic beads conjugated to tumor-related marker, such as CK, HER2, etc., to perform a second sorting on non-hematopoietic cells.

Previously, our negative magnetic enrichment was automated by PreCelleon Inc. However, a large part of this device is disposable, which brings high expense in testing one blood specimen. An optimized design of this device should be conducted to decrease the test cost.
8.2 Multiparameter Fluorescent Immunocytochemical Staining

The heterogeneity of CTCs requires multi-parameter analysis on the samples cells. In this research, methodologies of multi-parameter immunostaining were developed both for flow cytometry staining and slide staining.

Immunostaining of up to 9 colors was applied on CTCs for flow cytometry, in which the status of CD14, CD16, CD45, CD 68, CD115, HER2, EpCAM, CK and viability were tested. The populations of atypical tumor-related cells were found in flow plots of the enriched cells from metastatic breast cancers. The number of CK+, CD45+, EpCAM- cells which are beyond the conventional definition of CTC can correlate to patient survival significantly. The patients with 100- events on CK+ EpCAM- CD45- or CK+ EpCAM- CD45+ populations have better prognosis than the patients with 100+ events on these populations.

4-color immunocytochemical staining were applied to CTCs on slides and viewed with epifluorescence and confocal microscopy systems. The staining protocol was optimized to effectively label surface makers and intracellular markers, including some endonuclear proteins. CTCs positive on epithelial markers, mesenchymal markers, HER markers, Hh
pathway markers and cancer stem cell markers were detected by the staining of the enriched cells from cancer patient blood specimens. The visualization of these atypical CTCs in single cell resolution, including the CK+, CD45+, EpCAM- cells which were discussed in the flow cytometry analysis part, can present the expression and location of relevant proteins in heterogeneous CTCs.

In the respect of technology development, the future work in immunostaining can be focused on increasing the number of parameters. With quantum dots (QD) and Nuance multispectral camera, 6 parameters can be applied onto the same cell simultaneously. Currently, the usage of quantum dots has some limitations. Due to the particle size, QD can be resisted when delivered into cells. Surface modifications with peptides were reported to be able to help QD transmembrane transportation (Liu, 2011). These modified quantum dots should be used to stain with intracellular markers in CTCs. Beyond the color setting of 4-color staining, Qdot 585 and Qdot 655 may serve as two additional fluorochromes to reach 6 colors. Considering the relatively high brightness of quantum dots comparing with Alexa Fluor, optimization should be performed on typical markers on cell lines before conducting 6-color staining on CTCs.
Moreover, the outcome of 4-color immunostaining on the enriched cells should be analyzed quantitively to correlate with patients’ condition and prognosis. Previously, the γ-H2AX staining of CTCs aimed to evaluate the CTC DNA double strand breakage after the chemotherapy of ABT-888 in dose escalation trial. However, the manual numeration of CTCs positive on different markers brought heavy workload. An automated cell counting program combined with imaging system should be established to increase the throughput in CTC quantitative analysis.

8.3 Nucleic Acid in situ Hybridization on Tumor-related Gene

In addition to protein-level labeling, the nucleic acid in situ hybridization provides an insight in the extent of oncogene duplication and transcription. The DNA FISH can provide a highly quantitative result for CTC characterization. In the research, HER2 and EGFR gene abundance can be numerated on each CTC. The result deviates from pathological test on tissue, which shows genomic change in cell migration procedure.

The RNA ISH can analyze oncogene at transcription level with less background and non-specific binding than immunostaining. The high specificity of RNA ISH is proved by sense probe and negative control on HER2 and EGFR sequences, while the high
sensitivity of RNA ISH is proved by the detection of rare enzymatic transcripts in cell line. With our 4-color staining, mRNA transcript of HER family genes, Hh pathway markers, HPV viral genes, enzymatic genes and macrophage markers were found in the enriched cells from patient blood specimens.

The combined RNA ISH and ICC staining can analyze CTCs on both protein and RNA ISH levels. The combined staining can integrate the complementary superiorities of ICC staining and RNA ISH to study on heterogeneous CTCs at single cell resolution. The ICC staining can provide intense signals which allow a quick scan for potential tumor-related cells, while the RNA ISH, which is highly sensitive and specific, can analyze the cells quantitatively. Based on our 4-color staining setting, the combined RNA ISH and ICC staining was also extended to 6 color by using QD and Nuance camera.

Protocol for the combined RNA ISH and immunochemical staining was also developed for CTCs on flow cytometry. The reliability of the RNA ISH on flow cytometry has been proven by the spiking test of tumor cell lines and leukocytes on HER family markers. A small portion of events were found positive on HER family markers or HPV among the enriched cells from patient blood specimens on flow plots. This fact is in accordance with the rarity of CTCs.
Part of future research should be focused on the optimization of 5 or 6-color staining of combined RNA ISH and ICC staining. The emission intensity of ICC staining is 10+ folds higher than that of RNA ISH. The huge discrepancy of brightness can lead to incorrect spectral deconvolution in Nuance camera. This problem may be solved by either reducing the non-specific noise of ICC staining or selecting brighter fluorochromes in RNA ISH.

At the same time, for the markers of low expression or transcription in rare cells, the combined RNA ISH and ICC staining can provide high throughput screening with sensitive characterization. Some important weakly expressed markers can be the potential application for the staining methodologies, for example HPV-related transcripts. The impact of cell differentiation on HPV status was reported in the cell line or tissue sections in different cancer types (Wilson, 2005; Longworth, 2004). With our staining methodologies, the HPV markers, such as transforming protein E6, E7, transcription regulated protein E2 and structural protein L1, L2, can be studied on CTCs with different extents of differentiation.

To study the transformation from primary tumor to CTC, the similar 6-color staining protocol on tissue should be established. The matrix of tissue may bring additional
background noise to RNA ISH on weakly expressed mRNA transcripts, which needs to be solved in the future.

8.4 Microarray Analysis for Potential Marker Searching

To study CTCs with more relevant tumor markers to oncogenesis and metastasis for various tumors, microarray analysis was applied to perform high-throughput screening on tumor-related markers. Although microarray is not with single cell resolution, microarray can provide test up to 100+ markers for 96 cell samples within one test.

The Nanostring nCounter Expression Assays provides a robust multi-parameter high-throughput screening for 100-1000 magnetic enriched cells from patient blood specimens. Within 31 chosen potential CTC-related genes, besides some common oncogenes, BMI1, ERBB2IP, TGFB1, SERPINE1, and CXCR4 can be our future staining targets on CTCs. The centroid linkage, which is based on heatmap, shows the enriched tumor related cells from patient blood are very distant from cell lines and the enriched cells from healthy donors. The clustering shows the necessity for analyzing heterogeneous CTCs with single cell resolution.
The future work in microarray analysis can apply 3' IVT Expression Analysis with Human Genome U133 Plus 2.0 Array (Affymetrix) onto enriched cells. The Human Genome U133 Plus 2.0 Array can provide the expression test with over 47000 genes. Currently, the DNA quality of cells after magnetic separation and flow sorting is not adequate to 3' IVT Expression Analysis. Part of the reason is the DNA leakage in cell damage by the shear stress in flow cytometry and low DNA yield in DNA isolation kit. A better method to enrich the tumor-related cells and isolate the DNA should be developed.

The Quantigene Plex 2.0 Assay should also be applied for high throughput marker screening. The Quantigene Plex 2.0 shares the same amplification structure as the RNA ISH reported previously. The comparison between the results from Quantigene Plex 2.0 Assay and RNA ISH can show the difference in the results of analysis with single cell and total enriched cells.
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