Video Article

Adaptation of Semi-automated Circulating Tumor Cell (CTC) Assays for Clinical and Pre-clinical Research Applications

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URL: http://www.jove.com/video/51248
DOI: doi:10.3791/51248

Keywords: cancer, Metastasis, circulating tumor cells (CTCs), CellSearch system, user defined marker characterization, in vivo, pre-clinical mouse model, clinical research

Date Published: 10/16/2013

Citation: Lowes, L.E., Hedley, B.D., Keeney, M., Allan, A. Adaptation of Semi-automated Circulating Tumor Cell (CTC) Assays for Clinical and Pre-clinical Research Applications. *J. Vis. Exp.* (), e51248, doi:10.3791/51248 (2013).

Abstract

The majority of cancer-related deaths occur subsequent to the development of metastatic disease. This highly lethal disease stage is associated with the presence of circulating tumor cells (CTCs). These rare cells have been demonstrated to be of clinical significance in metastatic breast, prostate, and colorectal cancers. The current gold standard in clinical CTC detection and enumeration is the FDA-cleared CellSearch system (CSS).This manuscript outlines the standard protocol utilized by this platform as well as additional adapted protocols that describe the detailed process of user-defined marker optimization for protein characterization of patient CTCs, and a comparable protocol for CTC capture in very low volumes of blood, using standard CSS reagents,for study*ing in vivo* pre-clinical mouse models of metastasis. In addition, differences in CTC quality between healthy donor blood spiked with cells from tissue culture versus patient blood samples are highlighted. Finally, several commonly discrepant items that can lead to CTC misclassification errors are outlined. Taken together, these protocols will provide a useful resource for users of this platform interested in pre-clinical and clinical research pertaining to metastasis and CTCs.

Introduction

In 2013 it is estimated that 580,350 individuals will die from cancer and that 1,660,290 new cases of this disease will be diagnosed in the United States alone[1]. The majority of these deaths occur subsequent to the development of metastatic disease[2]. The current lack of effective therapies in treating metastases and a limited understanding of the metastatic cascade makes this stage of disease highly lethal{Formatting Citation}. The presence of circulating tumor cells (CTCs) within the bloodstream have been demonstrated to correlate with metastatic disease[3]. These cells are extremely rare and their detection isindicative of overall survival in metastatic breast[4]{Formatting Citation}, prostate[5]{Formatting Citation} and colorectal[6]{Formatting Citation} cancer. In these patients, the presence of ≥5 (breast and prostate) or ≥3 (colorectal) CTCs in 7.5 ml of blood is indicative of poorer prognosis when compared to those patients with fewer or no detectable CTCs in the same blood volume. In addition, the change in CTC number during or after therapeutic intervention has been demonstrated to be useful as a predictor of treatment response, often sooner than currently utilized techniques[7-10].

It has been estimated that, in metastatic cancer patients, CTCs occur at a frequency of approximately 1 CTC per 10^5-10^7 blood mononuclear cells and in patients with localized disease, this frequency may be even lower (~1 in 10^8).The rare nature of these cells can make it difficult to accurately and reliably detect and analyze CTCs[11]. Several methods (reviewed previously[12-14]) have been utilized to enrich and detect these cells by exploiting properties that differentiate them from surrounding blood components. In general, CTC enumeration is a two-part process that requires both an enrichment step and a detection step. Traditionally, enrichment steps rely on differences in physical properties of CTCs (cell size, density, deformability) or on protein marker expression (i.e., epithelial cell adhesion molecule [EpCAM], cytokeratin [CK]). Following enrichment, CTC detection can be performed in a number of different ways, the most common of which are nucleic acid-based assays{Formatting Citation}{Formatting Citation}{Formatting Citation}{Formatting Citation}{Formatting Citation} and/or cytometric approaches{Formatting Citation}. Each of these strategies are unique, having distinct advantages and disadvantages, {Formatting Citation}however they all lack standardization; a necessity for entrance into the clinical setting. The CellSearch system (CSS) was therefore developed to provide a standardized method for the detection and enumeration of rare CTCs in human blood using fluorescence microscopy and antibody-based techniques[4-6]. This platform is currently considered the gold standard in CTC enumeration and is the only technique approved by the U.S. Food and Drug Administration (FDA) for use in the clinic[15].

The CSS is a two component platform consisting of~~,~~ ***(1)*** the CellTracks AutoPrep system (hereafter referred to as the preparation instrument), which automates the preparation of human blood samples, and ***(2)*** the CellTracks Analyzer II (hereafter referred to as the analysis instrument),

which scans thesesamples following preparation. To distinguish CTCs from contaminating leukocytes the preparation instrument employs an antibody mediated, ferrofluid-based magnetic separation approach and differential fluorescence staining. Initially, the system labels CTCs using anti-EpCAM antibodies conjugated to iron nanoparticles. The sample is then incubated in a magnetic field, and all unlabeled cells are aspirated. Selected tumor cells are resuspended, and incubated in a differential fluorescence stain, consisting of fluorescently-labelled antibodies and a nuclear staining reagent. Finally, the sample is transferred to a magnetic cartridge, called a MagNest (hereafter referred to as the magnetic device), and scanned using the analysis instrument.

The analysis instrument is used to scan prepared samples using different fluorescence filters, each optimized to the appropriate fluorescent particle, using a 10X objective lens. CTCs are identified as cells that are bound by anti-EpCAM, anti-pan-CK-phycoerythrin (PE) (CK8, 18 and 19), and the nuclear stain 4', 6-diamidino-2-phenylindole (DAPI). Conversely, contaminating leukocytes are identified as cells that are bound by anti-CD45-allophycocyanin (APC) and DAPI. Following scanning, computer-defined potential tumor cells are presented to the user. From these images, the user must employ qualitative analysis using the defined parameters and differential staining discussed above to determine which events are CTCs.

In addition to providing a standardized method for CTC enumeration, the CSS allows for molecular characterization of CTCs based on protein markers of interest. This interrogation can be performed at the single-cell level, using a fluorescein isothiocyanate (FITC) fluorescence channel not required for CTC identification[16]. Although this platform provides the capacity for molecular characterization, the detailed process of protocol development and optimization is not well-defined. Threecommercially available markers have been developed by the manufacturer for use with the CSS, including epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), and insulin-like growth factor 1 receptor(IGF1R). HER2 analysis, in combination with the CSS, has been utilized by several groups to illustrate the potential for CTC characterization to inform clinical decision-making and to potentially change existing treatment guidelines. For example, Fehm et al. (2010) demonstrated that approximately one third of breast cancer patients with HER2-primary tumors had HER2+ CTCs[17]. In addition, Liu et al. (2013) recently reported that up to 50% of patients with HER+ metastatic breast cancer did not have HER2+ CTCs[18]. Herceptin, a HER2 recepter interfering monoclonal antibody demonstrated to greatly benefit patients whose tumors express sufficient levels of HER2, is a commonly utilized treatment for patients with HER2+ primary tumors[19-21]. However, these studies suggest that Herceptin may be being sub-optimally utilized and that CTC characterization may aid in predicting treatment response. Ultimately, CTC characterization may have the potential to improve personalized care.

CTC research is unique in that it has largely utilized a bedside-to-benchtop approach. This method, unlike benchtop-to-bedside research, which can often take years to impact patient care, has allowed CTCs quick entry into the clinical setting. However, physicians are hesitant to use results from CTC analysis in patient treatment decision-making due to a lack of understanding of their underlying biology. Therefore appropriate pre-clinical models of metastasis and complementary CTC analysis techniques must be utilized in order to investigate these outstanding questions. In general, there are 2 types of pre-clinical models used to study the metastatic cascade, ***(1)*** spontaneous metastasis models, which allow for the study of all the steps in the metastatic cascade, and ***(2)***experimental metastasis models, which only allow for the study of later steps in the metastatic process such as extravasation and secondary tumor formation[22]. Spontaneous metastasis models, involve tumor cell injections into appropriate orthotopic locations (e.g. injection of prostate cancer cells into the prostate gland for the study of prostate cancer). Cells are then given time to form primary tumors and spontaneously metastasize to secondary sites such as the bone, lung, and lymph nodes. In contrast, experimental metastasis models involve direct injection of tumor cells into the bloodstream (e.g. via tailvein or intracardiac injection to target cells to specific locations) and therefore skip the initial steps of intravasation and dissemination to secondary organs[22].Thus far the majority of CTC analysis in *in vivo* model systems has been performed using either cytometry-based[23] or adapted human-based CTC techniques (e.g., AdnaTest)[24]. Although useful, none of these techniques adequately reflect CTC enumeration using the gold standard CSS. Based on the clinical approval, standardized nature, and widespread usage of the CSS, the development of a CTC capture and detection technique for *in vivo* modeling that utilizes equivalent sample preparation, processing, and identification criteriawould be advantageous as results would be comparable to thoseobtained from patient samples. However, due to the volume requirements of the preparation instrument it is not possible to process small volumes of blood using this automated platform. In addition, previous work by Eliane et al., (2008) has demonstrated that contamination of samples with mouse epithelial cells (which also meet the standard CTC definition [EpCAM+CK+DAPI+CD45-]) can lead to misclassification of mouse squamous epithelial cells as CTCs[25]. To address these issues an adapted technique that allows the utilization of the CSS CTC kit reagents combined with a manual isolation procedure was developed. The addition of a FITC labelled human leukocyte antigen (HLA) antibody to the assay allows human tumor cells to be distinguished from mouse squamous epithelial cells.

This manuscript describes thestandard, commercially developed and optimized CSS protocol for processing patient blood samples and common pitfalls that may be encountered, including discrepant items that can lead to CTC misclassification errors. In addition, customization of the CSS assay to examine user-defined protein characteristics of captured CTCs and a comparable adapted CSS technique that allows for the enrichment and detection of CTCs from small volumes of blood in pre-clinical mouse models of metastasis are described.

Protocol

All human studies described in this manuscript were carried out under protocols approved by Western University's Human Research Ethics Board. All Animal studies were conducted in accordance with the recommendations of the Canadian Council on Animal Care, under protocols approved by the Western University Animal Use Subcommittee.

Standard CTC Enumeration from Patient Blood Samples using the CSS

1. Human Blood Sample Collection and Preparation for Processing on the Preparation Instrument

1. Using standard aseptic phlebotomy techniques, draw a minimum of 8.0 ml of human blood into a 10 ml CellSave tube (hereafter referred to as the CTC preservative tube), which contains ethylenediaminetetraacetic acid (EDTA) and a proprietary cellular preservative. Invert the tube 5 times to prevent blood from clotting. Samples may be processed immediately or stored at room temperature for up to 96 hours.
2. Remove CSSreagents from the fridge and allow them to warm to room temperature before using.
3. Using a disposable 10 ml pipette or automated pipettor, collect 7.5 ml of blood from the CTC preservative tube and slowly dispense blood into an appropriately labelled preparation instrument processing tube.
4. Add 6.5 ml of dilution buffer to each sample. Mix by inverting sample 5 times. Centrifuge sample at 800 x g for 10 min with the brake in the "off" position. Follow the on-screen instructions on the preparation instrument to load all patient samples onto the system for processing. Samples must be processed within 1 hour of preparation.

2. Control Preparation for Processing on the Preparation Instrument

1. Gently vortex the control vial and invert 5 times to mix.
2. Carefully remove the cap from the control vial and place an inverted preparation instrument processing tube on top of the uncapped vial. In one swift motion, invert the control vial, and pour the contents into the processing tube. While inverted, gently flick the sides of the control vial to release any remaining contents.
3. Carefully remove the inverted control vial from the processing tube, ensuring that no liquid is lost and place aside. Using a 1,000µl pipette, collect any remaining contents from the vial and lid and gently pipette into the processing tube.
4. Follow the on-screen instructions on the preparation instrument to load the control onto thesystem for processing.

3. Sample Scanning on the Analysis Instrument

1. Follow the on-screen instructions on the preparation instrument to unload all samples from the system. Loosely cap each magnetic device cartridge and tap the magnetic device using hands or lab bench to release any bubbles that are stuck to the edges of the cartridge.Once all the bubbles have been removed, firmly cap the cartridge, lay the magnetic device flat, and incubate in the dark for at least 20 minutes. Samples must be scanned within 24 hours of preparation.
2. Turn on the analysis instrument and initialize the lamp. Once warmed (~ 15 minutes), load the system verification cartridge onto the analysis instrument and select the *QC Test* tab. Follow the on-screen instructions to perform the necessary quality control measures.
3. Load a sample onto the analysis instrument and select the *Patient Test* tab. All saved information from the preparation instrument will be displayed. Click *Start* to initialize sample scanning. The system will perform a coarse focus and edge detection on the magnetic device cartridge.
4. Adjust all edges as necessary using the directional keys. Select *Accept*. The system will perform a fine focus and begin sample scanning.
5. Following control scanning the results should be validated using the defined criteria for cells spiked at high (CK+DAPI+CD45-APC+) and low (CK+DAPI+CD45-FITC+) concentrations.Following patient sample scanning the results should be reviewed for captured CTCs using the defined CTC criteria (CK+DAPI+CD45-).

CTC Characterization for User-Defined Markers using the CSS

1. Preparation of User-Defined Markersand Instrument Initialization

1. Dilute the antibody of interest using Bond Primary Antibody Diluent to the desired concentration in a marker reagent cup using the following formula, where the working concentration is the concentration of the antibody after addition to the sample and the stock concentration is the concentration of antibody in the reagent cup. For multiple samples, adjust the antibody volumes as described in **Table**. Place the marker reagent cup into position 1 in the reagent cartridge and load the cartridge onto CSS.
   Stock Concentration = Working Concentration x

   150µl
2. Collect blood, prepare samples, and load the preparation instrument as described in the above***Standard CTC Enumeration from Patient Blood Samples using the CSS*** protocol. To enable custom marker addition, select *User-Defined Assay* when prompted by the preparation instrument. Input the marker name and select a color. As samples are loaded onto the, the operator will be prompted to indicate which should receive custom marker by selecting *Yes* or *No* as necessary.

2. Sample Scanning of User-Defined Markers on the Analysis Instrument

1. Turn on the analysis instrument, initialize the lamp, and perform quality control and system verification as described in section 3.2 of the ***Standard CTC Enumeration from Patient Blood Samples using the CSS*** protocol.
2. Load a sample onto the analysis instrument and select the *Setup* tab. To initialize the FITC channel, select *CellSearch CTC* as the *Kit ID* under the *Test Protocols* section. From this menu, select *CTC Research*, click the *Edit* button and set the exposure time as desired. It is recommended that an exposure time of 1.0sec not be exceeded when using the CSS CTC kit as this can increase bleed-through into other fluorescent channels utilized for CTC identification.
   Adaptation of the Standard CSSProtocol for use in Pre-Clinical Mouse Mod

***Adapted from Veridex Mouse/Rat CellCapture Kit (no longer commercially available)*

1. Mouse Blood Collection and Storage

1. Prior to blood collection, run ~30µl of 0.5M EDTA back and forth through a 22 gauge needle, leaving a small amount of EDTA in the hub.
2. Collect a minimum of 50 µl of mouse blood from mice previously injected with human tumor cells via orthotopic, tail vein, or intracardiac routes. Collect blood from the saphenous vein (for serial CTC analysis) or by cardiac puncture (for terminal CTC analysis). Remove needle and dispense blood into a 1 ml EDTA microtainer blood collection tube. Mix by inversion or gently flick tube to prevent blood from clotting. Blood may be processed immediately or stored at room temperature for up to 48 hours following the addition of an equal volume of CytoChex cellular preservative.

2. CTC Enrichment

1. Remove CSS reagents from the fridge and allow them to warm to room temperature before using.
2. Transfer the equivalent of 50 µl of whole blood to a 12 x 75 mm flow cytometry tube. Add 500 µl of dilution buffer to each sample, washing down any blood that remains on the sides of the tube. If necessary, a short centrifuge spin can be used to collect any remaining blood.
3. Gently vortex the anti-EpCAM ferrofluid and add 25 µl to each sampleby placing the tip of the pipette directly into the sample. Add 25 µl of Capture Enhancement reagent and vortex gently to mix. Incubate samples at room temperature for 15 min.
4. Place sample tubes into the magnet and incubate for 10 min.While the sample tubes are still in the magnet, use a glass pipette to carefully aspirate the residual liquid without touching the wall of the tube next to the magnet and discard.

3. CTC Staining

1. Remove the sample tubes from the magnet and resuspend in 50 µl of Nucleic Acid Dye, 50 µl of Staining Reagent, 1.5 µl of anti-mouse CD45-APC, 5.0 µl of anti-human HLA-AlexaFluor488 and 100 µl of Permeabilization Reagent. For multiple samples, these reagents may be pre-mixed and 206.5 µl of mixture may be added to each tube. Vortex gently to mix and incubate for 20 min at room temperature.
2. Add 500 µl of dilution buffer, vortex gently, place sample tubes into the magnet, and incubate for 10 min.While the sample tubes are still in the magnet, use a glass pipette to carefully aspirate the residual liquid without touching the wall of the tube next to the magnet and discard. Remove the sample tubes from the magnet and resuspend in 350 µl of dilution buffer.Vortex gently to mix.

4. Magnetic Device Loading

1. Using a gel loading tip, carefully transfer the entire volume from the sample tube into a cartridge in the magentic device. Start at the bottom of the cartridge and slowly withdraw the tip as the sample is dispensed.
2. Once the entire sample has been transferred, loosely cap the magnetic device cartridge and tap the magnetic device using hands or lab bench to release any bubbles that are stuck to the edges of the cartridge as described in section 3.1 of the ***Standard CTC Enumeration from Patient Blood Samples using the CSS***.
3. Pop any bubblesusing a sterile 22 gauge needle by transferring them between the bevel and the edge of the cartridge. Once all the bubbles have been removed, firmly cap the cartridge, lay themagnetic device flat, and incubate in the dark for at least 10 minutes. Samples must be scanned within 24 hours of preparation.

5. Scanning of Manually Separated Samples on the Analysis Instrument

1. Turn on the analysis instrument, initialize the lamp, and perform quality control and system verification as described in section 3.2 of the***Standard CTC Enumeration from Patient Blood Samples using the CSS*** protocol.
2. Load the sample onto the analysis instrument and select the *Setup* tab. Clear any existing data on the magnetic device data button by clicking the *Format Sample* button. Enable the FITC channel and set the exposure time to 1.0 sec as described in section 2.2 of the ***CTC Characterization for User-Defined Markers using the CSS***.
3. Click on the *Patient Test* tab and select *Edit* to input the sample information. Select *CellSearch CTC* as the *Kit ID* and *CTC Research* as the *Test Protocol*. Input the remaining necessary information as indicated as the asterisk. *Save* the sample information and click *Start*.

Representative Results

Standard CTC Enumeration Assay

The sensitivity and specificity of the CSS has been well documented in the literature. However, to validate equivalent CTC recovery, spiked (1,000 LNCaP human prostate cancer cells) and unspiked human blood samples from healthy volunteer donors were processed on the CSS using the standard CSSCTC protocol. The expected unspiked samples were free of CTCs, 0.00±0.00%, and CTC recovery was demonstrated to be 86.9±4.71% for spiked samples(**Figure 1A**). CSS gallery images obtained from spiked samples were of optimal quality and CTCs were easy to distinguish from non-CTCs. However, when processing samples obtained from cancer patients, identification of CTCs is slightly more challenging, with many cells appearing smaller in size and being less easily distinguishable from non-CTCs **(Figure 1B)**. In addition, when reviewing patient samples 6 categories of events were identified that were commonly discrepant items between reviewers **(Figure 1C)**. These 6 categories included, ***(1)*** small events that did not meet the 4µm size requirement for CTC classification; ***(2)***items with dim CK and/or DAPI staining; ***(3)*** justified (should be counted as a CTC)versus unjustified (should not be counted as a CTC) bleed through into the CD45-APC channel caused by bright CK-PE staining; ***(4)***FITC events; ***(5)*** pixelated images in the CK and/or DAPI channels; and ***(6)***events with DAPI staining that is larger than CK images or those with DAPI staining that does not overlap >50% with the CK image. For categories ***(2)*** and

(5) specific criteria exist for CTC classification. For categ items with dim CK/DAPI can be classified as CTCs provided that an intact membrane can be observed in the CK channel and an appropriately sized DAPI image pted. For category ***(5)***, items with pixelated CK/DAPI cannot be classified as CTCs if *any* pixelation is observed in the CK channel. However, pixelation is acceptable in the DAPI channel provided that it is not too severe (i.e., image is entirely white on a background, no grey areas—described by Janssen Diagnostics (formerly Veridex) as white paint on a black background) or diffuse (must still appear oval in shape and fit within the CK).

User-Defined Marker Assay Development

Adaptation of the CSS to characterize CTCs for user-defined markers requires significant work-up with rigorous controls and has been described previously[16]. As a general rule, appropriate optimization of any user-defined marker requires that negative controls be employed to ensure that results are specific. The best results are obtained when spiked samples are processed with both a non-specific IgG control in place of the target antibody and with the antibody diluent alone as described previously. Various target antibody concentrations and exposure times should also be assessed and validated using cell lines with high, low, and absent(ne e) antigen densities. Optimal protocol conditions are achieved when the assay demonstrates both high sensitivity for the target antigen and low background noise from non-specific binding[16].

An example of this work-up using a cancer stem cell marker, CD44, is presented here. Initial testing with this marker began using the standard CSS CTC kit (hereafter referred to as the traditional CTC kit), which utilizes the FITC channel for user-defined marker development. Using the traditional CTC kit,it was demonstrated that, after significant optimization, the maximum number of CTCs that could be classified as CD44+ was 69.3±2.67% using samples spiked with 1,000 MDA-MB-468 human breast cancer cells, known to demonstrate high CD44 expression with the majority of cells (98.4±0.90%; as determined by flow cytometry) expressing this protein **(Figure 2A)**. Based upon these findings it was hypothesized that the commercially available CSS CXC kit might produce improved results. This kit allows for improved visualization of markers with a lower antigen density (~50,000 antigens/cell) compared with the traditional CTC kit (optimized for markers with a density of ~100,000 antigens/cell) by reversing the fluorescence channel in which the CK8/18/19 (traditionally represented in the PE channel) and the user's marker of interest (traditionally represented in the FITC channel) are represented (therefore hereafter the CXC kit will be referred to as the low antigen density CTC kit)[26]. After significant optimization, it was demonstrated that this change allowed for improved CD44 ing, with 98.8±0.51% of CTCs classified as CD44+ using CD44-PE at a concentration of 1.0µg/ml and an exposure time of 0.6s**(Figure 2A)**. Appropriate optimization of a er-defined marker also requires validation using high antigen density (MDA-MB-468), low antigen density (21NT), and negative (LNCaP l lines for the marker of interest **(Figure 2B)**.

CTC Analysis in Pre-Clinical Mouse Models

To determine the sensitivity and specificity of the adapted mouse CSS protocol, spiked (1,000 LNCaP human prostate cancer cells) and unspiked mouse blood samples were processed manually and scanned on the analysis instrumentand compared to results obtained using the same cell line processed using the standard automated CSS protocol on the preparation instrument**(Figure 3A)**. As expected, unspiked samples were free of CTCs using both assays, 0.00±0.00% and CTC recovery using the adapted mouse kit (90.8±5.18%) was not significantly different from results obtained using the standard automated system (86.9±4.71%; $p > 0.05$). Images obtained using the manual mouse adapted protocol did not differ from those observed using the standard automated technique, with the exception of the addition of the HLA-FITC marker. In addition, mouse squamous epithelial cells do not stain positively for HLA-FITC **(Figure 3B)**. To confirm that this technique was as sensitive as the standard CSS protocol for the isolation of low number of CTCs,serial dilutionswere performed with spiked blood s es and the correlation of expected number of cells versus recovered number of cells was assessed **(Figure 3C)**. Results demonstrate thatC s could effectively be recovered down to a sensitivity of 5 cells per 50µl of whole blood using this assay. These values correlated with expected results with an r^2 = 0.99.

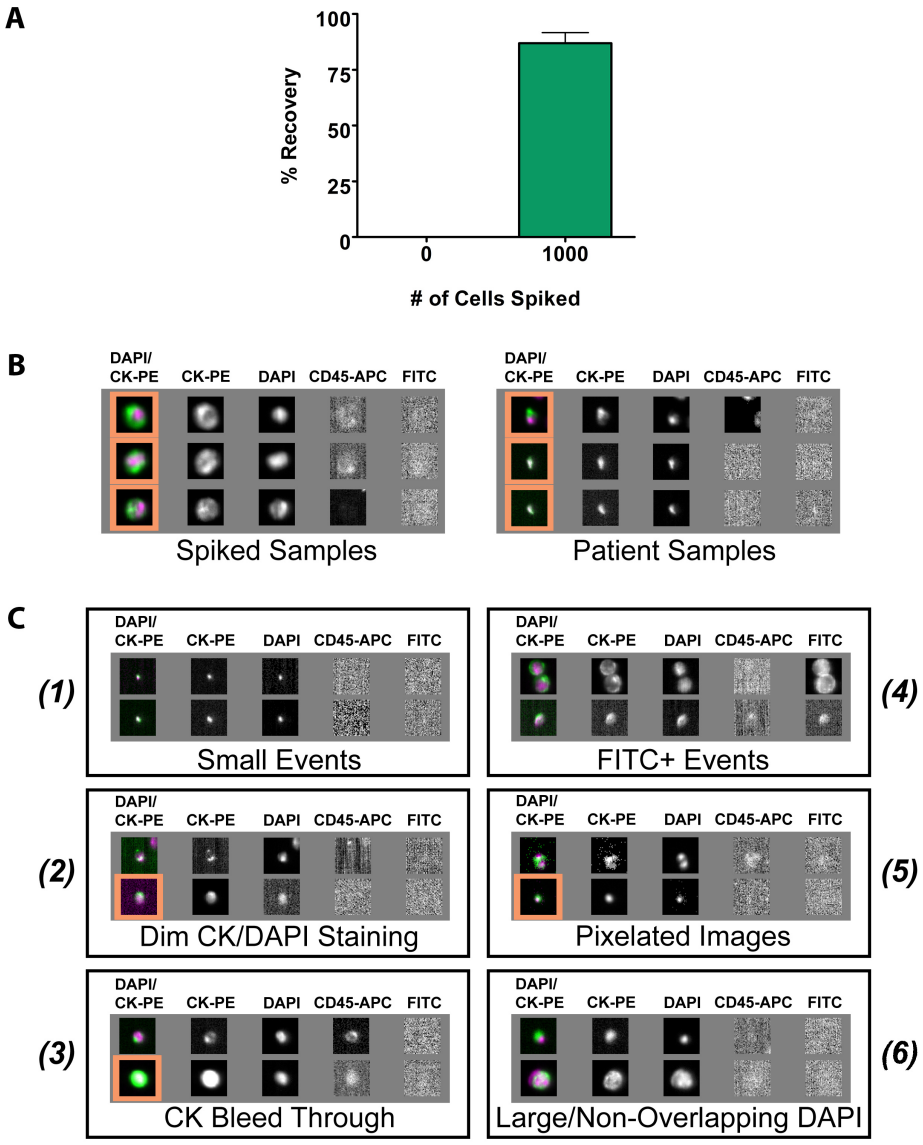

Figure 1: CTC enumeration and interpretation using the standard CSS protocol. (A) CTC recovery measured as a percentage of the number of spiked cells. Cells were counted by hemocytometer and ~1,000 LNCaP human prostate cancer cells were spiked into 7.5ml of human blood. Unspiked human blood samples were used as a negative control (n=3). Data are presented as the mean ± St... **(B)** Representative CSS gallery images of the differences in CTC quality observed in spiked blood samples (i.e., healthy donor blood spiked with tumor cells from culture) versus samples collected from cancer patients. **(C)** Representative CSS gallery images of commonly discrepant items that are often misclassified. Orange squares indicate acceptable CTCs, identified as CK+/DAPI+/CD45-. Images acquired at 10x objective magnification.

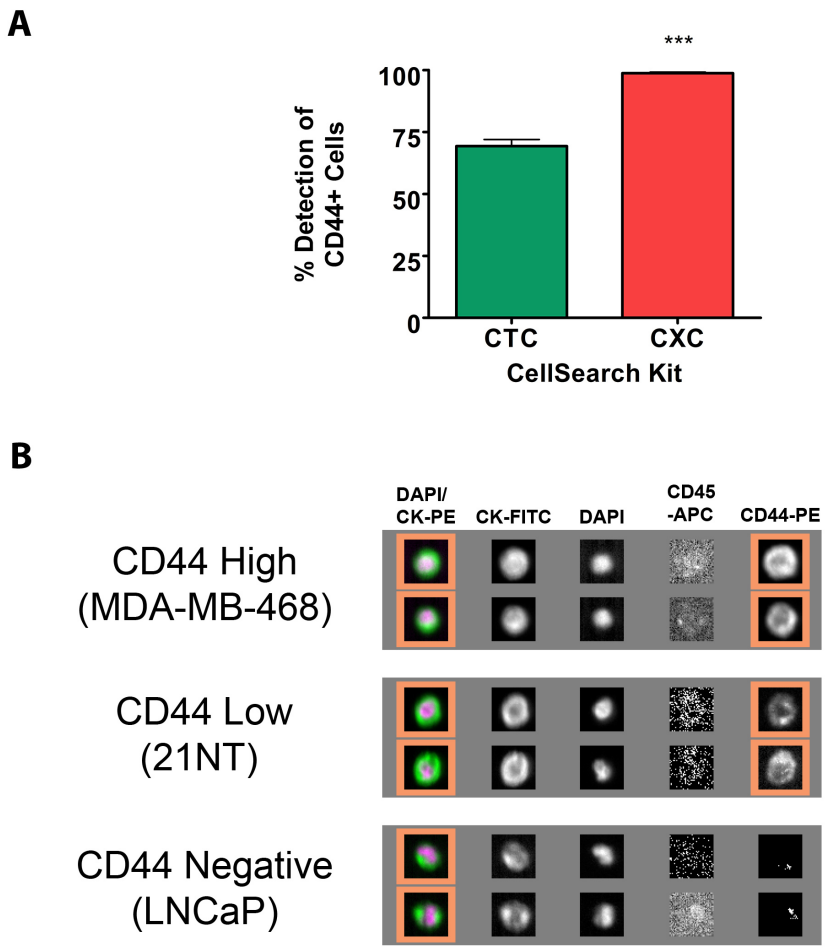

Figure 2: Characterization of CTCs for user-defined markers using the CSS. (A) Percentage of cells classified as CD44+ using the [illegible] and CXC kits on the CSS (n=3). Data are presented as the mean ± SEM. *** = significantly different (P < 0.0005). **(B)** Representative CS gallery images of blood from a healthy volunteer donor (7.5ml), spiked with ~1,000 cells from the identified cell line, incubated with 1.5µg/ml of anti-CD44-PE, and scanned at an exposure time of 0.2s. Orange squares indicate CD44+ CTCs, identified as CK+/DAPI+/CD45-/CD44-PE+.

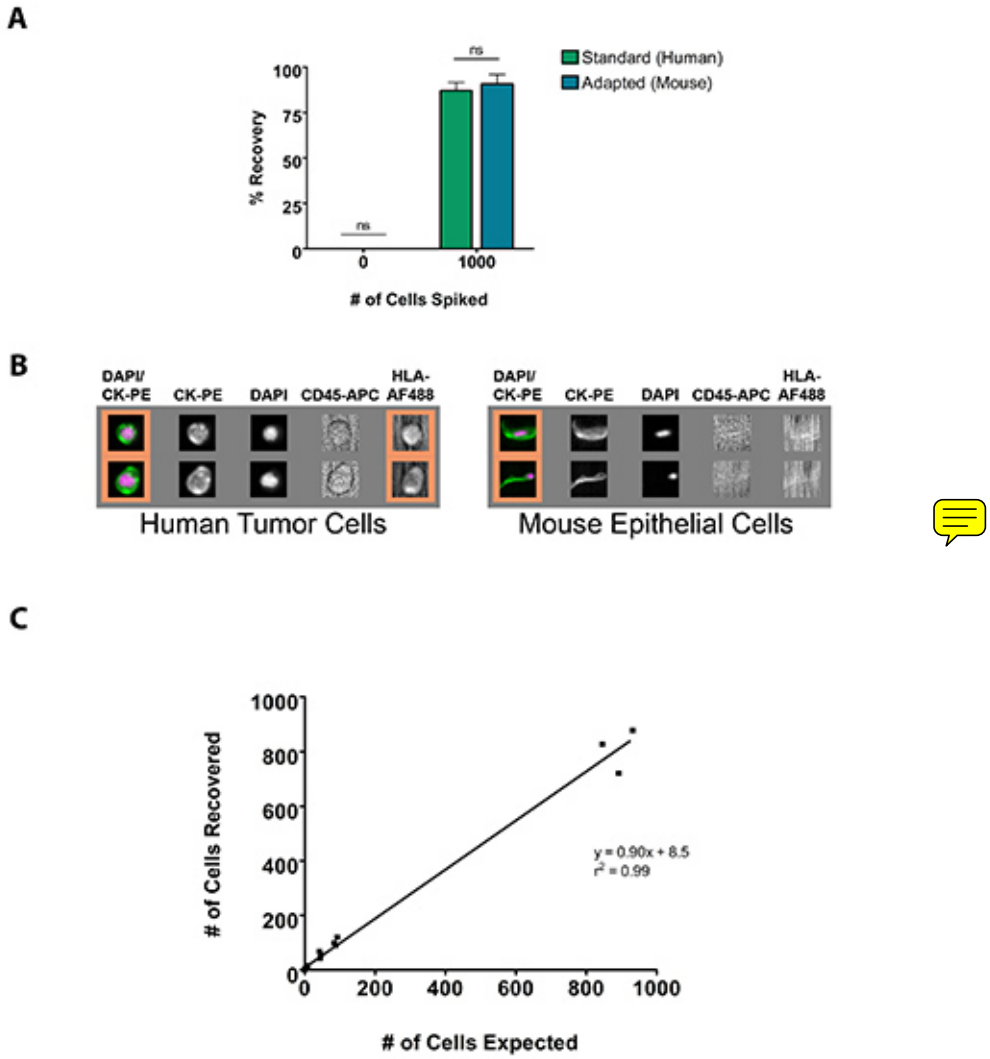

Figure 3: Adaptation of the CSS procedure for use in pre-clinical mouse models of metastasis. (A) CTC recovery using the adapted mouse CSS protocol measured as a percentage of the number of spiked cells and compared to results obtained using the standard human CSS protocol. Cells were counted by hemocytometer and ~1,000 LNCaP human prostate cancer cells were spiked into 7.5ml of human blood. Unspiked human blood samples were used as a negative control (n=3). Data are presented as the mean ± SEM. ns = not significant.**(B)**Representative CSS gallery images of CTCs captured the adapted mouse CSS protocol demonstrating that HLA-AF488 is able to distinguish human from mouse cells.**(C)**Analysis of correlation and linear regression of the expected versus recovered number of spiked tumor cells at various concentrations. LNCaP human prostate cancer cells were initially counted by hemocytometer and subsequently spiked into mouse blood at a concentration of ~1,000 tumor cells/50µl of blood. Spiked mouse blood was then serially diluted to a concentration of 5 tumor cells/50µl and processed using the mouse adapted CSS protocol (n=3).

# of Samples with User Defined Marker Added	Total Volume to Add to Reagent Cup (µl)
1	450
2	600
3	750
4	900
5	1050
6	1200
7	1350
8	1500

Table 1: Total volume requirements for the CSS when processing various numbers of samples with a user-defined marker.

Discussion

Despite the development of many new CTC technologies since the introduction of the CSS in 2004, this technique is still the only clinically approved technology on the market today and therefore it is considered the current gold standard for CTC detection and enumeration. This manuscript has demonstrated that although the CSS has rigorous quality control standards it can be subject to interpretation bias and that CTC identification in patient samples is much different from identification in spiked samples. Six categories of commonly discrepant items were identified that can cause CTC misclassifications to occur. These discrepant items highlight the need for multiple reviewers on each patient sample processed on this instrument. In addition, the differences observed in spiked versus patient obtained CTCs demonstrates that there is a necessity for any new CTC technologies to be validated in both spiked and patient samples. In addition, these new technologies must be compared to the gold standard CSSusing split sample testing of both spiked and patient samples, as efficient CTC capture from spiked samples only does not necessarily reflect CTC capture efficiency in patient samples.

Although the CSS has the capability to perform characterization of captured CTCs, it is quite restricted with regards to highly customizable optimization. In general, the only parameters that can be changed on this instrument for optimization of user-defined markers are the antibody concentration and the length of time that the fluorophore is exposed to the mercury lamp. This limited capacity for optimization can present problems when working-up user-defined markers on the CSS. One solution proposed in this manuscript (described in detail previously[16]) is the use of the low antigen density CTC kit which switches the FITC and PE fluorescent channels allowing for better visualization of markers with a low antigen density. Regardless of which kit is utilized (traditional- or low antigen density CTC kit) there are several necessary steps that must be undertaken to ensure appropriate marker sensitivity, specificity, and optimization. Firstly, assay sensitivity must be assessed in comparison to a well validated method, such as flow cytometry, that will allow determination of the expected detection level (i.e., the % of cells in the cell population that express the marker of interest) of the user-defined marker. Secondly, the assay must be assessed for its ability to detect the marker of interest in cell lines with various levels of expression (i.e., high and low antigen densities) and its specificity must be validated in a cell line that is negative for the marker of interest. In all cases, all cell lines must be tested using a cells only control (no antibody added), the appropriate IgG control, and the antibody of interest at various concentrations and exposure times to determine the most appropriate settings that will ensure optimization of the user-defined marker. However, it should be noted that although characterization of CTCs is possible on the CSS, currently only one user-defined marker of interest can be explored in each sample, and that the system is very limited with regards to downstream applications due to the harsh processing of the samples.

The unique bench-side-to-bench approach utilized in CTC research has allowed for quick entry of this useful assay into the clinical setting.However, it has resulted in inadequateunderstanding of the biology of these rare cells. Therefore the development and optimization of assays that allow for assessment of CTCs in pre-clinical *in vivo* mouse models of cancer are needed. This manuscript describes an adapted CSS protocol that allows for CTCs to be assessed in 50µl volumes of mouse blood, ideally suited for serial CTC collection experimentation. This manuscript demonstrates that CTC enumeration using this protocol is comparable to results obtained using the CSS in combination with the traditional CTC kit, with no significant differences in CTC capture efficiency between the automated and manual separation techniques. In addition, during the development of this assay it was recognized, as previously described in the literature[27], that mouse squamous epithelial cell contamination can make accurate identification of CTCs more difficult and sometimes impossible. Therefore to combat this issue a user-defined HLA-AlexaFluor488 was added to this protocol to ensure that only human cells (CK+DAPI+CD45-HLA-AlexaFluor488+) are being appropriately assigned as CTCs. It is important to note that the LNCaP cell line used in this manuscript is HLAlow and therefore HLA-AlexaFluor488 may need to be titrated for cell lines with varying HLA expression. Although we have added an HLA-AlexaFluor 488 to our protocol to ensure accurate identification of CTCs, it is noteworthy that the vast majority of mouse squamous epithelial cells were easily identifiable by morphology and were limited in cell number(0.33±0.24 events/50µl; n=9). Higher cell numbers(up to 11 events/50µl) were only observed when blood collection (via cardiac puncture) was improved difficult and repeated attempts were necessary. Therefore we propose that if desired, on-system characterization of CTCs could be accomplished by omitting this marker. In addition, although not described here, we anticipate that collection of and further downstream characterization of CTCsusing other methods could be easily achieved as demonstrated previously using the CSS[27,28].

Although the CSShas been utilized clinically to effectively detect CTCs in the blood of metastatic breast, prostate and colorectal cancer patients[4-6], it does have several limitations. In up to 35% of patients with various metastatic cancers, CTCs are undetectable despite the presence of widespread systemic disease[30]. This lack of detection has been proposed to be as a result of the epithelial-to-mesenchymal transition, a well-documented process known to enhance cancer invasion, metastasis, and overall aggressiveness[31]. This transition has been associated with a significant reduction in epithelial markers, such as EpCAM, and a corresponding increase in mesenchymal markers[32]. Several studies have recently demonstrated that the presence of these mesenchymal markers in CTCs are predictive of poorer prognosis and that many of these cells lack expression of epithelial markers that would be necessary for their detection using the CSS[24,33-38]. This suggests that the standard CSS definition may be missing some of the most aggressive CTCs.

Despite the described limitations, it is anticipated the protocols described in this manuscript will be important tools for improved CTC analysis using the CSS, development of novel CTC technologies, optimization of user-defined markers, and improved understanding of CTC biology using *in vivo*pre-clinical mouse models. Taken together, these protocols will provide a useful resource for users of this platform interested in pre-clinical and clinical research pertaining to metastasis and CTCs.

Disclosures

The author (A.L.A.) received funding that was provided by Janssen Oncology, whose parent company Johnson & Johnson also owns Janssen Diagnostics LLC, which produces reagents and instruments used in this article.

Acknowledgements

This work was supported by grants from the Ontario Institute of Cancer Research (#08NOV230), the Canada Foundation for Innovation (#13199), Prostate Cancer Canada, Janssen Oncology, the London Regional Cancer Program, and donor support from John and Donna Bristol through the London Health Sciences Foundation (to A.L.A.). L.E.L. is supported by a Frederick Banting and Charles Best Canada Graduate Scholarship Doctoral Award. A.L.A is supported by a CIHR New Investigator Award and an Early Researcher Award from the Ontario Ministry of Research and Innovation.

References

1. Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2013. *CA: a cancer journal for clinicians***63** (1), 11-30, doi:10.3322/caac.21166 (2013).
2. Chambers, A. F., Groom, A. C. & MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. *Nature reviews. Cancer***2** (8), 563-72 (2002).
3. Pantel, K. & Brakenhoff, R. H. Dissecting the metastatic cascade. *Nature reviews. Cancer***4** (6), 448-56, doi:10.1038/nrc1370 (2004).
4. Cristofanilli, M., Budd, G. T., *et al.* Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *The New England journal of medicine***351** (8), 781-91 (2004).
5. De Bono, J. S., Scher, H. I., *et al.* Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research***14** (19), 6302-9, doi:10.1158/1078-0432.CCR-08-0872 (2008).
6. Cohen, S. J., Punt, C. J. a, *et al.* Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO***20** (7), 1223-9, doi:10.1093/annonc/mdn786 (2009).
7. Hayes, D. F., Cristofanilli, M., *et al.* Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clinical cancer research : an official journal of the American Association for Cancer Research***12** (14 Pt 1), 4218-24, doi:10.1158/1078-0432.CCR-05-2821 (2006).
8. Budd, G. T., Cristofanilli, M., *et al.* Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research***12** (21), 6403-9, doi:10.1158/1078-0432.CCR-05-1769 (2006).
9. Olmos, D., Arkenau, H.-T., *et al.* Circulating tumour cell (CTC) counts as intermediate end points in castration-resistant prostate cancer (CRPC): a single-centre experience. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO***20** (1), 27-33, doi:10.1093/annonc/mdn544 (2009).
10. De Bono, J. S., Scher, H. I., *et al.* Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research***14** (19), 6302-9 (2008).
11. Allan, A. L. & Keeney, M. Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. *Journal of oncology***2010**, 426218 (2010).
12. Lowes, L. E., Goodale, D., Keeney, M. & Allan, A. L. Image cytometry analysis of circulating tumor cells. *Methods in cell biology***102**, 261-90, doi:10.1016/B978-0-12-374912-3.00010-9 (2011).
13. Lianidou, E. S. & Markou, A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. *Clinical chemistry***57** (9), 1242-55, doi:10.1373/clinchem.2011.165068 (2011).
14. Alix-Panabières, C. & Pantel, K. Circulating tumor cells: liquid biopsy of cancer. *Clinical chemistry***59** (1), 110-8, doi:10.1373/clinchem.2012.194258 (2013).
15. Parkinson, D. R., Dracopoli, N., *et al.* Considerations in the development of circulating tumor cell technology for clinical use. *Journal of translational medicine***10**, 138, doi:10.1186/1479-5876-10-138 (2012).
16. Lowes, L. E., Hedley, B. D., Keeney, M. & Allan, A. L. User-defined protein marker assay development for characterization of circulating tumor cells using the CellSearch® system. *Cytometry. Part A : the journal of the International Society for Analytical Cytology***81** (11), 983-95, doi:10.1002/cyto.a.22158 (2012).
17. Fehm, T., Müller, V., *et al.* HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. *Breast cancer research and treatment***124** (2), 403-12 (2010).
18. Liu, Y., Liu, Q., *et al.* Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. *BMC cancer***13**, 202, doi:10.1186/1471-2407-13-202 (2013).
19. Slamon, D. J., Leyland-Jones, B., *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *The New England journal of medicine***344** (11), 783-92, doi:10.1056/NEJM200103153441101 (2001).
20. Marty, M., Cognetti, F., *et al.* Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology***23** (19), 4265-74, doi:10.1200/JCO.2005.04.173 (2005).
21. Slamon, D., Eiermann, W., *et al.* Adjuvant trastuzumab in HER2-positive breast cancer. *The New England journal of medicine***365** (14), 1273-83, doi:10.1056/NEJMoa0910383 (2011).
22. Welch, D. R. Technical considerations for studying cancer metastasis in vivo. *Clinical & experimental metastasis***15** (3), 272-306at <http://www.ncbi.nlm.nih.gov/pubmed/9174129> (1997).
23. Goodale, D., Phay, C., Postenka, C. O., Keeney, M. & Allan, A. L. Characterization of tumor cell dissemination patterns in preclinical models of cancer metastasis using flow cytometry and laser scanning cytometry. *Cytometry. Part A : the journal of the International Society for Analytical Cytology***75** (4), 344-55 (2009).
24. Gorges, T. M., Tinhofer, I., *et al.* Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC cancer***12**, 178, doi:10.1186/1471-2407-12-178 (2012).
25. Eliane, J.-P., Repollet, M., *et al.* Monitoring serial changes in circulating human breast cancer cells in murine xenograft models. *Cancer research***68** (14), 5529-32, doi:10.1158/0008-5472.CAN-08-0630 (2008).

26. Veridex White Pages. Available at: http://www.veridex.com/pdf/CXC_Application_Guideline.PDF. (2008).
27. Flores, L. M., Kindelberger, D. W., *et al.* Improving the yield of circulating tumour cells facilitates molecular characterisation and recognition of discordant HER2 amplification in breast cancer. *British journal of cancer* **102** (10), 1495-502 (2010).
28. Sieuwerts, A. M., Kraan, J., *et al.* Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast cancer research and treatment* **118** (3), 455-68, doi:10.1007/s10549-008-0290-0 (2009).
29. Cohen, S. J., Punt, C. J. a, *et al.* Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **26** (19), 3213-21, doi:10.1200/JCO.2007.15.8923 (2008).
30. Allard, W. J., Matera, J., *et al.* Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10** (20), 6897-904 (2004).
31. Thiery, J. P., Acloque, H., Huang, R. Y. J. & Nieto, M. A. Epithelial-mesenchymal transitions in development and disease. *Cell* **139** (5), 871-90, doi:10.1016/j.cell.2009.11.007 (2009).
32. Yang, J. & Weinberg, R. a Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Developmental cell* **14** (6), 818-29 (2008).
33. Gradilone, A., Raimondi, C., *et al.* Circulating tumour cells lacking cytokeratin in breast cancer: the importance of being mesenchymal. *Journal of cellular and molecular medicine* **15** (5), 1066-70, doi:10.1111/j.1582-4934.2011.01285.x (2011).
34. Königsberg, R., Obermayr, E., *et al.* Detection of EpCAM positive and negative circulating tumor cells in metastatic breast cancer patients. *Acta oncologica (Stockholm, Sweden)* **Epub** (Epub), Epub (2011).
35. Kasimir-Bauer, S., Hoffmann, O., Wallwiener, D., Kimmig, R. & Fehm, T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. *Breast cancer research : BCR* **14** (1), R15, doi:10.1186/bcr3099 (2012).
36. Mego, M., Mani, S. A., *et al.* Expression of epithelial-mesenchymal transition-inducing transcription factors in primary breast cancer: The effect of neoadjuvant therapy. *International journal of cancer. Journal international du cancer* **Ep** (Epub), Epub (2011).
37. Yu, M., Bardia, A., *et al.* Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science (New York, N.Y.)* **339** (6119), 580-4, doi:10.1126/science.1228522 (2013).
38. Armstrong, A. J., Marengo, M. S., *et al.* Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. *Molecular cancer research : MCR* **9** (8), 997-1007, doi:10.1158/1541-7786.MCR-10-0490 (2011).