Enrichment of Circulating Tumor Cells from a Large Blood Volume Using Leukapheresis and Elutriation: Proof of Concept

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Background: The aim of this study was to determine the applicability of a sequential process using leukapheresis, elutriation, and fluorescence-activated cell sorting (FACS) to enrich and isolate circulating tumor cells from a large blood volume to allow further molecular analysis.

Methods: Mononuclear cells were collected from 10 L of blood by leukapheresis, to which carboxyfluorescein succinimidyl ester prelabeled CaOV-3 tumor cells were spiked at a ratio of 26 to 10⁶ leukocytes. Elutriation separated the spiked leukapheresates primarily by cell size into distinct fractions, and leukocytes and tumor cells, characterized as carboxyfluorescein succinimidyl ester positive, EpCAM positive and CD45 negative events, were quantified by flow cytometry. Tumor cells were isolated from the last fraction using FACS or anti-EpCAM coupled immunomagnetic beads, and their recovery and purity determined by fluorescent microscopy and real-time PCR.

Results: Leukapheresis collected 13.5×10^9 mononuclear cells with 87% efficiency. In total, 53 to 78% of spiked tumor cells were pre-enriched in the last elutriation fraction among 1.6×10^9 monocytes. Flow cytometry predicted a circulating tumor cell purity of ~90% giving an enrichment of 100,000-fold following leukapheresis, elutriation, and FACS, where CaOV-3 cells were identified as EpCAM positive and CD45 negative events. FACS confirmed this purity. Alternatively, immunomagnetic bead adsorption recovered 10% of tumor cells with a median purity of 3.5%.

Conclusions: This proof of concept study demonstrated that elutriation and FACS following leukapheresis are able to enrich and isolate tumor cells from a large blood volume for molecular characterization. © 2010 International Clinical Cytometry Society

Key terms: circulating tumor cell; enrichment; leukapheresis; elutriation; flow cytometry; ovarian cancer; circulating tumor cell enrichment and isolation; fluorescence-activated cell sorting

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In the US and Europe 85,000 women are diagnosed annually with ovarian cancer, 75% of which already exhibit advanced disease stage due to an asymptomatic course and the lack of suitable markers (1–4). Even after cytoreduction and chemotherapy 25% of patients relapse within the first 6 months (5), and 75% of patients are expected to succumb to the disease within 5 years. Radiological examination and cancer antigen 125 serum level dynamics are used to detect disease recurrence, even though these methods lack both sensitivity and specificity. This indicates the urgent need for new, more Additional Supporting Information can be found in the online version of this article.

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efficient methods and better biomarkers to identify ovarian cancer and disease recurrence at an early state to improve patient survival and quality of life. In ovarian cancer, disease recurrence is a result of either localized metastasis to the adjacent peritoneal cavity, or distant metastasis to nonadjacent organs and bone marrow via the lymphatic system and circulation. Distant metastases have been identified in 40% of ovarian cancer patients over the course of disease, the majority of which take root prior to or within the first 2 years after initial diagnosis (6,7). Circulating tumor cells (CTCs) are the principle vehicle of distant metastasis, detaching from the tumor mass and entering the peripheral blood even at early disease stage (8–11).

There exist two main challenges to the isolation and detection of CTCs: (1) their low abundance in blood, on the order of 1 CTC/mL (12) or 1 CTC per 10^6 - 10^7 leukocytes (13), and (2) the lack of a known feature that uniquely identifies them. CTCs have generally been enriched based upon their physical character by their density using gradient centrifugation (9,14,15) and by their large size using micro-filtration (16-19), or immunologically using antibodies targeting such as the epithelial cell adhesion molecule (EpCAM) that are immobilized on substrates including microfluidic chambers and magnetic beads for CTC adsorption (10,12,20). The enrichment of CTCs using gradient centrifugation and/ or immunomagnetic bead adsorption revealed a CTC incidence rate of 20% in ovarian cancer patients at primary diagnosis. In late stage disease, the incidence rate increased to between 37 and 57% with a median detection of 0.25 to 1 CTC per mL of blood (12,21,22). Approximately 90% of ovarian cancers are epithelial in origin (23). In an attempt to detect and enumerate CTCs following enrichment, fluorescent microscopy is commonly used with antibodies specific for epithelial cells including cytokeratin and EpCAM with or without the combination of a nuclear stain and CD45 for negative staining of leukocytes. Although enrichment by these techniques facilitates the detection of CTCs using subsequent fluorescent microscopy, they are not sufficient to isolate them due to the large background of leukocytes that persist reducing the purity of CTCs to 1% or less (14,20). The use of a functional assay to actively bind and separate CTCs from peripheral blood mononuclear cell (PBMNC) enriched samples via a cell adhesion matrix resulted in an increased median CTC count of 31.5 CTCs per mL of blood. This technique also revealed an increasing CTC level and incidence from early to late stage disease (10% of 10 patients with a mean of 6 CTCs per mL versus 73% of 52 patients with a mean of 41 CTCs per mL). Matrix bound CTCs were found to form colonies proving their viability and proliferative capacity in long term culture, and patients with CTCs had a poorer disease free survival compared with those without CTCs (11). Regardless of the enrichment method used, CTC yield and incidence is limited by the volume of blood able to be drawn from patients for analytical purposes, which rarely exceeds 50 mL. This is further compromised by the use of current enrichment techniques that typically have a capacity of 10 mL of blood or less per trial.

A process is presented here with the capability to interrogate the entire blood volume of a patient for the enrichment and isolation of CTCs using three sequential semi-automated systems: (1) leukapheresis, (2) counterflow centrifugal elutriation, and (3) flow cytometry driven fluorescence-activated cell sorting (FACS) (Fig. 1). Leukapheresis is a routine density centrifugation system used to harvest PBMNCs by circulating the total blood volume of a patient up to 3 times. This system has been used without complication for patients with advanced disease stage and age receiving autologous transplantation of CD34 positive cells (13,24), or mobilized dendritic cells for immunotherapeutic purposes following primary tumor resection and high dose chemotherapy (25). When quantified, a median of 2 to 7 CTCs per million PBMNCs were observed in 20% of leukapheresates harvested from metastatic patients consistent with other enrichment techniques (13,26-28). Elutriation following

leukapheresis has been used to separate leukocytes based primarily on their size to enrich and purify monocytes for dendritic cell tumor immunotherapy (25,29-32). CTCs are anticipated to be enriched along with monocytes due to their large size and comparable density (33,34) simplifying their subsequent detection and isolation. Final enrichment and isolation of tumor cells was performed using FACS, or immunomagnetic bead adsorption, which currently represents the commonest form of CTC enrichment in practice. FACS was chosen as a high throughput system that permits the quantification and sorting of rare events in real-time using multiple parameters (markers as well as physical characteristics) for the positive selection of CTCs and elimination of contaminating leukocytes simultaneously (8,35). This study was used as a proof of concept to determine the applicability and efficiency of elutriation combined with FACS following leukapheresis for the enrichment and isolation of viable CTCs with a high degree of purity. This in turn would enable a robust molecular analysis of this rare and enigmatic cell population.

MATERIALS AND METHODS Leukocyte Harvest from Healthy Donors

Leukapheresis was performed using the COBE[®] Spectra system and auPBSC program (software version 6.1, Caridian BCT, Lakewood, CO) to collect PBMNCs. Leukapheresates were obtained from seven healthy male donors aged 20 to 50 with informed written consent, and the experimental protocol was approved by the Medical University of Vienna Ethics Commission. For effective elutriation the leukapheresate must contain greater than 10^9 monocytes within 5 to 30×10^9 PBMNCs. To meet the specified requirements, the donor was connected to the system by dual venipuncture and 10 L of blood were circulated at a rate of 65 mL/min under a continuous flow centrifugation of 2,400 rpm (maximum of 910g). Anticoagulant (Acid Citrate Dextrose Solution A, Baxter, Deerfield, IL) was delivered at a 1:12 ratio and 16.5 mM of ionic calcium (4 \times 2.23 mmol ampule, Fresenius Kabi, Graz, Austria) in 0.9% NaCl solution (freeflex[®], Fresenius) was infused at a rate of 125 mL/h to mitigate hypocalcemia. On average, 14.0 \pm 3.8 \times 10⁹ leukocytes were harvested from the seven donated leukapheresates. A pre and postleukapheresis blood cell count was determined by the CELL-DYN System (Abbott Diagnostics, Abbott Park, IL). After collection, leukapheresates were stored overnight with shaking (22°C, 50-60 Hz), and diluted to a final volume of 300 mL with PBS (pH 7.2, Baxter, Deerfield, IL) immediately prior to elutriation.

Tumor Cell Culture and Spiking

The ovarian epithelial tumor cell line CaOV-3 (ATCC HTB-75TM) was chosen because they express EpCAM at high density facilitating their detection and isolation after enrichment, and have been found to range in diameter consistent with what has been reported for CTCs

(19,34). The cell line was purchased from the American Type Culture Collection (ATCC) and authenticated by short tandem repeat (STR) genotyping as described below. CaOV-3 cells were cultured in Cellstar® tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) under standard conditions at 37°C with 95% relative humidity and 5% CO₂ in growth medium consisting of Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with 4.5 mg/L glucose, 10% FBS (Sigma, Taufkirchen, Germany), and 1% penicillinstreptomycin (Sigma, St. Louis, MO). Cultures were harvested at 90% confluence by incubation with 0.25% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA, Sigma) for 5 min at 37°C. According to manufacturer's instructions, 10^6 cells were prelabeled with 1.5 μ M carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Eugene, OR) and resuspended in 1 mL of PBS with 1% FBS (staining buffer). To determine the viable cell count, two replicates of 100,000 CFSE labeled cells were incubated with propidium iodide (2 µg/mL, Sigma) for 5 min at 4°C, placed into TruCOUNTTM tubes (BD Biosciences, San Jose, CA) containing fluorescent beads, and 50,000 CFSE positive events were acquired using a FACSCaliburTM flow cytometer and analyzed using CellQuest PRO software (BD Biosciences). The average cell count was used to spike 26 (n = 6) or 2.6 (n = 1) tumor cells for every 10⁶ leukocytes into the leukapheresates.

Pre-Enrichment of Tumor Cells by Elutriation

The Elutra[®] system (Caridian BCT) was programmed to enrich monocytes providing 5 or 6 distinct leukocyte fractions using either the default or modified version, respectively. The modified version was provided by the company during the experimentation period (white paper 306670-247, Caridian BCT). According to Stokes' Law, cells are separated by counter-flow centrifugal elutriation into distinct fractions by their size and to a lesser extent density based on their sedimentation velocity. Briefly, cells enter a conical chamber under a constant centrifugal force. Fluid is then introduced in the opposite direction at a flow rate which produces an equilibrium force causing cells to align based upon their size. Cells of similar specific gravity may then be separated according to their differential size by sequentially increasing the counter fluid flow rate with smaller cells elutriating first. In this study, leukapheresates were fractionated under a constant centrifugation rate of 2,400 rpm (maximum of 500 to 900g from top to bottom of chamber) for both the default (37, 97.5, 103.4, 103.9, and 103.9 mL/min) and modified (37, 74, 97, 100, 101, and 101 mL/min) programs using the incremental counter-fluid flow rates indicated in parentheses. The objective of each program was to recover at least 60% of monocytes with 80% purity in the last elutriated fraction. Prior to elutriation, a 200 µm pore filter (Transfusion Set, Meditrade Medicare, Kufstein, Austria) was added to the leukapheresate inlet-line to eliminate gross cell aggregates. Each fraction was collected in ~ 1 L of elutriation buffer composed of PBS with 1% human

serum albumin (50 g/L Human Albumin, Baxter). The last elutriation fraction was collected in 250 mL of elutriation buffer with the rotor off.

Leukocyte Distribution

The absolute leukocyte count and distribution within the leukapheresate and elutriation fractions was quantified by flow cytomtery. An aliquot of 100,000 leukocytes was transferred to a TRUCOUNT tube and stained using an antibody cocktail containing fluorochrome-conjugtated monoclonal antibodies against CD45 FITC (clone 2D1, 1.6 µg/mL), CD15 FITC (clone HI98, 1:50), CD56 PE (clone NCAM16.2, 0.4 µg/mL), CD3 PerCP (clone SK7, 0.4 µg/mL), CD19 APC (clone SJ25C1, 0.8 µg/mL), and CD14 APC (clone MΦP9, 1.6 µg/mL) for 30 min at 4°C (20). For each sample 50,000 CD45 positive events were acquired. Cells were similarly stained with appropriate isotype-matched IgG antibodies conjugated with FITC, PE, PerCP-Cy5.5 and APC (clone MOPC-21, 1:10) to serve as negative controls. All antibodies were obtained from BD Biosciences. Red blood cell and platelet counts were determined by CELL-DYN.

Tumor Cell Detection by Flow Cytometry

Sample preparation and flow cytometry were performed in accordance with best practices established for the analysis of rare events (36-40). One million leukocytes from each elutriation fraction and the tumor cell spiked leukapheresate were distributed in triplicate to BD FalconTM tubes (polystyrene, BD Biosciences), centrifuged at 350g for 7 min at 22°C, and resuspended in 50 µL of staining buffer. The cells were then double stained for 30 min at 4°C with anti-EpCAM PerCP-Cy5.5 (clone EBA-1, 1:10) for bivariate identification of tumor cells and the pan-leukocyte marker anti-CD45 APC for the negative selection of leukocytes (clone HI30, 1:5) (BD Biosciences). After staining, samples were diluted 1:10 in FACS lysing buffer (1 to 1.5% formaldehyde, BD Biosciences) for cell fixation and to lyse remaining red blood cells. The entire sample was then acquired by a FACSCaliburTM flow cytometer at a rate of 500 to 1,000 events/s and analyzed using CellQuest PRO software (BD Biosciences). To increase the signal to noise ratio, a total cell trigger using a FSC threshold was set to exclude the majority of debris and damaged cells. Color compensation for each staining combination was performed using single-stained tumor cells and leukocytes, and confirmed using dual-stained populations. Nonspecific binding of leukocytes to epithelial cell-specific antibodies and vice versa was evaluated using cells stained with the appropriate isotype-matched IgG fluorochrome conjugated antibodies, which also acted as negative controls. As an additional negative control, 10⁶ leukocytes from the leukapheresate were similarly labeled and analyzed prior to tumor cell spiking to determine the level of background noise. Tumor cells were then classified as CFSE positive, EpCAM positive and CD45 negative events. After gating based upon these criteria, nonspecific fluorescence was further reduced using a predefined tumor cell region within the forward scatter (FSC) vs. side scatter (SSC) dot plot based upon the size and internal complexity of tumor cells from control samples. To estimate cell viability following enrichment, a sample from the last elutriation fraction was additionally labeled with propidium iodide and diluted 1:10 in staining buffer just prior to acquisition.

Immunological Isolation of Tumor Cells

Similar to flow cytometry detection, 10^8 leukocytes from the last elutriation fraction were transferred to a FACS tube in 500 μ L of staining buffer (n = 2), incubated with anti-EpCAM PerCP-Cy5.5 (1:1) and anti-CD45 APC (1:1) for 30 min at 4°C, washed and resuspended in 2 mL of staining buffer. Events were sorted into two groups, either CD45 positive or CD45 negative, after gating based upon their expression of CFSE and EpCAM and FSC vs. SSC profile using a FACSAriaTM cell sorter and FACSDiVaTM software (BD Biosciences). The entire sample was collected at a rate of 20,000 to 30,000 events/s and sorted events were collected in 500 µL growth medium maintained at 4°C. To ensure a high tumor cell purity, the anticoincidence mode (Purity Sort Precision Mode) was enabled to accept sorting units (droplets) containing only target cells, with the additional condition that the leading and trailing half-unit also be void of contaminating nontarget cells. Alternatively, magnetic beads coupled with anti-EpCAM antibody via a DNA linker were used to isolate and purify tumor cells from the last elutriation fraction (n = 4) (CEL-LectionTM epithelial enrich Dynabeads[®], Miltenyi, Auburn, CA). According to the manufacturer's instructions, 10^7 leukocytes were incubated in triplicate with 4 \times 10⁷ beads. Bound cells were then magnetically separated from the suspension, and subsequently eluted from the beads by incubation with 400 IU/mL of DNase-I.

Tumor Cell Detection by Fluorescent Microscopy

One million leukocytes from the leukapheresate and the last elutriation fraction, immunomagnetic bead eluted cells and \sim 500 cells from each FACS sorted group were prepared in triplicate and cytocentrifuged onto adhesive slides at 2,000 rpm for 5 min (Thermo Fischer Scientific, Shandon EZ Cytofunnels[®], Cytoslides[®] and Cytospin[®] 4, Waltham, MA). Cells were air dried for 20 min, blocked with 10% goat serum (Invitrogen) in PBS for 20 min at 22°C, and labeled with anti-EpCAM (clone 323/A3), anti-epidermal growth factor receptor (EGFR, clone H11), and anti-human epidermal growth factor receptor 2 (HER-2/neu, clone 9G6.10) for 10 min at 22°C (2.5 µg/mL, NeoMarkers, Freemont, CA). Primary antibody binding was revealed by incubation with AF546 goat anti-mouse (4 µg/mL Invitrogen) for 15 min at 4°C. Slides were then fixed with 3% paraformaldehyde (Sigma) in PBS for 15 min at 37°C, and permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 10 min at 22°C prior to staining with anti-pan-cytokeratin FITC (10 µg/mL, clone CK3-6H5, Invitrogen) for 30 min at 22°C and Hoechst 33258 dye (1 µg/mL, Invitrogen), after which slides were mounted with Fluoromount- G^{TM} solution (Southern Biotech, Birmingham, AL). Immunofluorescent detection was carried out using a CelÎR fluorescent imaging station with an Olympus BX51 confocal microscope (Olympus, Essex, UK) at $100 \times$ magnification.

DNA Extraction

High molecular weight DNA was extracted using the AllPrepTM DNA/RNA Micro kit with QIA Shredder 250 Tubes (Qiagen, Valencia, CA). PBMNCs used for control samples during DNA analysis were resolved by Ficoll density gradient centrifugation (Biocoll Separating Solution, 1.077 g/mL, Biochrom AG, Berlin, Germany). Briefly, 10 mL of whole blood were diluted 1:1 with PBS containing 1% FBS and 2.7 mM EDTA, layered directly onto the gradient solution and centrifuged at 800g for 30 min at 22°C with no brake. The buffy coat was collected, washed twice with dilution buffer at 400g and 350g for 10 min at 22°C and resuspended in 1 mL of PBS.

Real-Time PCR

The quantity of male leukocyte DNA was determined using the DYS14 assay as described by Zimmerman et al. (41), and of total cellular DNA (80 to 500 cells) using the Assay-On-DemandTM HS01395736_S1 (Chromosome 3) and HS01686063_S1 (Chromosome 11) (Applied Biosystems or ABI, Foster City, CA). All probes were 6-carboxyfluorescein (FAM) labeled, and all samples were prepared in triplicate. TaqMan[®] real-time PCR was performed under standard conditions employing the ABI PRISM 7900 Analyzer HT Sequence Detection System (ABI) (41). A standard calibration curve, generated by mixing leukocytes obtained from male donors with CaOV-3 tumor cells at representative dilutions (1, 5, 10, 20, 50, 60, 70, 80, 90% PBMNCs), was used to calculate the percent concentration of leukocytes corresponding to the ratio of male to total cellular DNA in FACS sorted samples.

Genotyping

The identity of CaOV-3 tumor cells and leukocytes from control and FACS sorted samples (~30 or 120 total cells) was verified and/or determined using seven of the nine ATCC STR loci D5S818, D7S820, D13S317, D16S539, CSF1-PO, TPOX, and vWA (Sigma Genosys, Saint Lious, MO) as described in the PowerPlex[®] 16 System Technical Manual (Promega, Madison, WI) with the exception that 50 amplification cycles were used. The anti-sense primers from each pair were fluorescently labeled with Cy5. Denatured PCR products along with an external standard DNA sizer (ALFexpress Sizer: 50, 100, 150, 250, and 300 bp, Amersham Biosciences, Piscataway, NJ) were resolved on an 8% polyacrylamide gel (High Resolution Reprogel, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in $0.5 \times$ TBE buffer (10× composition: 890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA, pH 8.3) using an automated laser fluorescence

DNA sequencer (ALFexpressTM II, Pharmacia, Uppsala, Sweden). The run was performed overnight at 1,500 V, 60 mA, and 30 W with a sampling interval of 2 s. The STR DNA fragment size was calculated using the external standard by the Fragment ManagerTM Software (AM software v3.01, Pharmacia), and the corresponding allele determined using the STR Factsheet (National Institute of Standards and Technology Short Tandem Repeat DNA Database, www.cstl.nist.gov/biotech/strbase/ str fact.htm).

CaOV-3 Cell Size Measurements

An aliquot of CaOV-3 cells for each trial was diluted 1:1 in 0.5% trypan blue and placed on a hemocytometer for light microscopy. Digital images (four unique regions yielding \sim 100 cells) were captured using the CellB imaging station and software (Olympus) under 400× magnification, and the cell diameter was determined from area measurements.

Statistical Analysis

The average of triplicate samples performed during flow cytometry detection and immunomagnetic bead adsorption was used to report tumor cell recovery, enrichment and purity for each trial. Data are represented as mean \pm standard deviation. The mean monocyte recovery, purity and enrichment, and tumor cell recovery and enrichment obtained by either the default or modified elutriation programs were compared using a two-sided *t*-test for independent samples. Multiple regression was used to evaluate the association between log transformed leukocyte and CD45 positive and EpCAM positive event counts. Statistical analysis was carried out using SPSS 15.0 (SPSS Inc, Chicago, IL) with a significance level of 0.05.

RESULTS

Leukapheresate Composition

To harvest at least one billion monocytes 10 L of blood were circulated by leukapheresis over a 3 h period. This process successfully harvested 87% of total blood volume PBMNCs equal to on average 13.5×10^9 PBMNCs from 5.2 L of blood concentrated in 100 mL of 90% platelet depleted plasma. The majority of leukocytes harvested by leukapheresis were PBMNCs, 83% of which were lymphocytes and 17% monocytes resulting in the collection of 1.2 to 3.3 billion monocytes for each trial. The 97% reduction of granulocytes by leukapheresis resulted in approximately a two to threefold enrichment of PBMNCs. The leukapheresates also contained 14×10^{10} platelets and 26.5×10^9 red blood cells (99.9% reduction from blood). The leukocyte composition of the leukapheresate and the last elutriation fraction for each trial is given in Supporting Infomation Table 1.



Fig. 2. Distribution of lymphocytes, monocytes, and tumor cells as a percent of the total elutriated for each distinct population plotted against elutriation fraction number using the (A) default (Trials 1 to 3) or (B) modified (Trials 4 to 7) monocyte enrichment program.

Cell Distribution by Elutriation

Elutriation was used to separate the tumor cell spiked leukapheresates into five or six distinct cellular fractions. Each elutriation required ~60 min. Platelets, constituting the smallest blood cell component, were completely removed in the first elutriation fraction, and red blood cells were completely eliminated prior to the last fraction. During elutriation 94% of lymphocytes were collected in fractions 2 and 3 using the default program and in fractions 2 through 4 using the modified program with a purity of 93%. The distribution of PBMNCs and tumor cells during elutriation is depicted in Figure 2. No statistical difference in monocyte or tumor cell enrichment in the last elutriation fraction was found between the default and modified programs (P >0.2 regardless of parameter compared), thus permitting the evaluation of the process using their combined values. However, the modified program indicated a trend for reduced variation and greater reliability compared with the default program, which failed to distribute monocytes in the last fraction during two experiments caused by an overaggressive flow rate (data not shown). In total, 97% of monocytes were recovered from the leukapheresate during elutriation, 77% of which were collected with 81% purity in the last fraction. This corresponded to 1.6 ± 0.6 billion monocytes enriched approximately fivefold as compared with the leukapheresate in this fraction. In three of the seven trials a greater number of monocytes were found after elutriation than existed in the leukapheresate (TruCount and CELL-DYN counts). Quality control measurements indicated that the aggregation between monocytes and platelets observed in the leukapheresate were not preserved after elutriation, suggesting that the discrepancy was a result of monocyte FSC vs. SSC shift and

reduced capacity to bind antibody in the leukapheresates (data not shown). Granulocytes were also collected primarily in the last elutriation fraction.

Tumor Cell Quantification by Flow Cytometry Detection

CFSE prelabeled CaOV-3 tumor cells were spiked at a ratio of 26 to 1 million leukocytes into the leukapheresate, and their distribution and recovery by elutriation was determined using flow cytometry analysis as shown in Figure 3. CaOV-3 cells used in this study were observed to have a diameter ranging from 15.9 to 34.7 μ m (median and mean of 22 μ m) consistent with what has been observed for CTCs (33,34). Because of their large size, $85\% \pm 15\%$ of tumor cells were found in the last elutriation fraction corresponding to a spiked tumor cell recovery of $66\% \pm 8\%$ therein (Table 1). No tumor cell events were observed in leukapheresate samples obtained prior to tumor cell spiking indicating a specificity of 100% for the described flow cytometry detection method. Events which were EpCAM positive and CD45 negative were defined as CTCs to determine their expected purity and enrichment after FACS as follows:

$$\% \text{ Purity} = \frac{\text{CFSE}^+ \setminus \text{EpCAM}^+ \setminus \text{CD45}^- \text{events}}{\text{EpCAM}^+ \setminus \text{CD45}^- \text{events}} \bullet 100\% (1)$$

Enrichment by Elutriation FACS =
$$\frac{\% \text{ Purity}/100}{\text{Spike Ratio}}$$
 (2)

By this definition, the expected CTC purity determined by flow cytometry analysis was 58% \pm 21% giving an enrichment of CTCs by elutriation and FACS of 2.4 \pm 0.9 \times 10⁴ and a total enrichment of 6.6 \pm 2.2 \times 10⁴



Fig. 3. The distribution and recovery of tumor cells by elutriation was analyzed by flow cytometry with cellular events contoured onto dot plots based upon their size (FSC), granularity (SSC) and CFSE, EpCAM and CD45 signal intensities. Individual samples from Trial 7 represent according to row: (A) CaOV-3 tumor cells labeled with CFSE and anti-EpCAM antibody, (B) leukocytes from the leukapheresate (without tumor cells) labeled with anti-EpCAM anti-EpCAM antibody, (B) leukocytes from the leukapheresate (without tumor cells) labeled with anti-EpCAM and anti-CD45 antibodies, and (C) the last elutriation fraction, in which a total of 818,621 CD45 positive events and 102 tumor cell events were identified during acquisition. Tumor cells were classified as CFSE and EpCAM positive and CD45 negative events (orange), and the leukocyte count was determined by CD45 expression represented by the CD45 histogram in Row B. Each tumor cell event was then morphologically confirmed to be within the FSC vs. SSC Tumor Cell Region. CD45 vs. EpCAM dot plots only contain CFSE and EpCAM positive gated events. No tumor cell events were found in the leukapheresate samples. $98.5\% \pm 1.5\%$ of events positive for CFSE were also positive for EpCAM indicating that these events represented intact tumor cells having a high EpCAM expression density. The viability of tumor cells prior to spiking, 96.3%, and after elutriation, 94.6%, was not found to be different further proving that the process is well tolerated by CaOV-3 cells.

including leukapheresis. Backgating of EpCAM positive and CD45 negative events based upon predetermined tumor cell physical characteristics (FSC vs. SSC tumor cell region in Fig. 3) further increased the expected purity to 92% \pm 6%, and thus the total enrichment to 10.8 \pm 3.9 \times 10 4 (Table 1).

Interestingly, a CD45 positive population was observed that was also positive for EpCAM at expression

Detection											
	Tumor cells per 106 leukocytes		Tumor cell enrichment		Recovery (%)						
Trial	Spiked	Recovered	Elutriation	Total (104) ^a	Total elutriated	Spiked	Purity (%)				
1	21	35	1.7	9.74	75	68	88				
2	2.6	20	7.8	70.3	55	65 (13)	82 (1)				
3	26	174	6.7	9.0	93	64 (5)	97 (32)				
4	26	136	5.2	18.4	92	53 (15)	96 (8)				
5	26	90	3.5	10.0	94	78 (9)	93 (2)				
6	26	74	2.8	7.6	98	74	91				
7	26	111	4.2	9.8	91	60	97				

 Table 1

 Quantification of Spiked Tumor Cell Enrichment, Recovery and Purity in the Last Elutriation Fraction by Flow Cytometry

 Detection

Statistical analysis does not include Trial 2 because a 10-fold lower spike ratio was used.

Values given in parentheses refer to tumor cell isolation by immunomagnetic beads.

^aEnrichment by leukapheresis, elutriation, and FACS compared with the total number of nucleated blood cells of the donor.

intensities consistent with tumor cells. The highest number of such events were found in the leukapheresate, and decreased with increasing elutriation fraction. Multiple regression revealed that, of the leukocyte populations, only B-lymphocytes were significantly associated with EpCAM binding contributing to a 2.45-fold increase in CD45 positive and EpCAM positive events with every 10-fold increase in B-lymphocytes (B = 0.39, P = 0.024). This observation provides strong support for the use of leukocyte lineage specific markers for negative selection to ensure a high specificity during CTC detection.

Tumor Cell Enrichment and Isolation

Tumor cells were immunologically isolated from the last elutriation fraction using FACS. A total of 79,120,000 and 76,376,941 CD45 positive events were acquired by the two FACS experiments yielding 1,272 and 3,301 CD45 negative and 4,889 and 7,948 CD45 positive sorted events after gating for intracellular CFSE and EpCAM expression. Thus, on average 20% of spiked tumor cells were recovered in the CD45 negative group after FACS. Fluorescent microscopy indicated a tumor cell purity of 94.1% in the CD45 negative sorted samples consistent with flow cytometry detection, and 24.0% in the CD45 positive sorted samples (Fig. 4). Using this purity, it can be estimated that 15% of spiked tumor cells were recovered in the CD45 positive sorted group. Although the aggregation of leukocytes with tumor cells was directly observed in the CD45 positive FACS sorted group, the number of aggregates was low compared with the number of individual leukocytes detected. The PBMNC concentration in the CD45 negative and CD45 positive groups were determined to be 15.5% and 82.9%, respectively, using real-time PCR, in agreement with fluorescent microscopy. To eliminate the possibility that female DNA may have been derived from another source, the identity of CaOV-3 tumor cells before and after enrichment was confirmed by STR genotyping. In three of the seven loci tested (D7S820, D16S539 and CSF1PO), CaOV-3 tumor cells could also be distinguished from PBMNCs. Alternatively, tumor cells from the last elutriation fraction were isolated by EpCAMcoupled magnetic beads and their recovery and purity determined by fluorescent microscopy. A mean number of 4, 128, 378, and 100 tumor cells was counted for the four independent trials giving a tumor cell recovery of $10.5\% \pm 4.4\%$ with a median purity of 3.5% (Table 1).

DISCUSSION

Isolation of CTCs is compromised by their low abundance in the circulation and the absence of specific morphological features or markers that uniquely identify them. In this study, a three-step sequential process was developed in an attempt to enrich and isolate CTCs from a large blood volume based upon their physical characteristics by (1) leukapheresis and (2) elutriation, and immunologically by (3) FACS. Current enrichment techniques are restricted by the low amount of blood that can be processed requiring sensitive detection methods to identify only a few CTCs within a large background of leukocytes (12,18,42-45). The use of leukapheresis to collect CTCs from cancer patients has the propensity to limit CTC escape and increase the amount of CTCs isolated by subsequent methods. Leukapheresates were used as the starting blood cell product for spiking experiments due to the logistic difficulty to obtain large donations of whole blood. In the study model, tumor cells from the ovarian cancer cell line CaOV-3 were spiked into leukapheresates prior to separation by elutriation, and tumor cell recovery was determined by flow cytometry. Since flow cytometry has an expected resolution of one epithelial tumor cell in 10^5 nucleated cells (46,47), a spike ratio of 26 tumor cells per million PBMNCs (~50 tumor cells per mL of blood) was specified to ensure their detection. The large number of cells harvested by leukapheresis necessitated the physical pre-enrichment of CTCs to allow the practical use of immunological based methods for their isolation thereafter. Elutriation is able to separate up to 30 billion leukocytes with similar density into distinct fractions based upon their square diameter in a single 1 h trial. CTCs contained in the leukapheresate would be



Fig. 4. Fluorescent microscopy images representative of CaOV-3 tumor cells and leukocytes from the last elutriation fraction that were either isolated by FACS into CD45 negative or positive sorted groups, or captured and eluted by EpCAM coupled immunomagnetic beads. Cells were counted at $\times 100$ magnification, and classified as tumor cells if they stained positive for Hoechst (nuclear), cytokeratin (cytoskeletal) and at least one of the membrane antigens EpCAM, EGFR or HER-2. Individual stains are represented in the first three columns, and are merged in the last column. Tumor cells were morphologically confirmed by their large size, rounded appearance, and high nuclear to cytoplasmic ratio. Tumor cells were clearly distinguished from leukocytes using these criteria. The FACS CD45 positive images present a tumor cell to leukocyte aggregate. Arrow depicts bead captured cells prior to elution. Images were acquired at $\times 400$ magnification (scale bar = 50 µm).

similarly separated without incurring size dependent loss, and enriched in each elutriation fraction according to Eqs. 1 and 2. Pre-enrichment by elutriation reproducibly recovered 53 to 78% of spiked tumor cells in the last fraction with a coefficient of variation of 12%. A similar recovery was observed in one trial when the spike ratio was decreased 10-fold. This trial acted as a probe to determine whether tumor cells could be detected at expected CTC levels in patient samples (11,12,22). Tumor cell loss associated with the method of detection is considered to be minimal since flow cytometry has an expected sensitivity of greater than 90% at acquisition rates of less than 2,000 events/s (35). In combination this led to a physical 10-fold enrichment due to the depletion of granulocytes by leukapheresis and lymphocytes during elutriation. As a result, $\sim 240,000$ tumor cells were distributed along with 1.6 billion monocytes with a purity of 80% in the last elutriation fraction. The removal of the vast majority of lymphocytes thought to bind to EpCAM (20), and granulocytes known to contribute false positive events during subsequent molecular analysis, has the potential to improve the specificity of subsequent immunological enrichment techniques (48-50). Alternative physical enrichment techniques such as micro-filtration and density centrifugation may substitute the elutriation process; however, both techniques have capacity based limits not exceeding 30 mL of blood or 15 mL of buffy coat per test increasing the handling of large cell products that may result in cell loss. Simple micro-filtration gives the highest depletion of leukocytes, but the capture of CTCs is dictated by the membrane pore size, which may lead to decreased sensitivity (16). OncoQuick gradient centrifugation is able to enrich CTCs 100-fold greater than elutriation, but within a mixed leukocyte population (Greiner Bio-One) (14,51).

Physical separation methods are sufficient to serve as a pre-enrichment step to significantly deplete leukocytes, but due to their low specificity CTCs are obtained

Parameter	Density gradient (15)	μ-Filtration (19)	Magnetic Bead (12,20)	Microfluidics (10)	Elutriation & FACS				
Enrichment Method	Oncoquick®	ISET (8 μm pores)	CellSearch [™] (anti-EpCAM)	CTC-chip (anti-EpCAM)	Elutriation & FACS				
Cytometric detection	CK ⁺ /hematoxylin ⁺	CK ⁺ /7AAD ⁺	CK ⁺ /DAPI ⁺ /CD45 ⁻	CK ⁺ /DAPI ⁺ /CD45 ⁻	CFSE ⁺ /EpCAM ⁺ /CD45 ⁻				
Spike ratio (CTC/mL ^a)	7	3	3-150	50-50,000	26 ^b				
Capacity (mL ^a /test)	15	1×12 wells	7.5	1-2	TBV				
Enrichment factor	4.5×10^{2}	$5 imes 10^{3c}$	4.0×10^{4}	7.1×10^{4c}	10.8×10^4				
Recovery (%)	55	80	85	61	66				
Purity (%)	0.04	0.2	1.4	50	90				

Table 2 Comparison of Loading Circulating Tumor Coll (CTC) Enrichment Techniques

CK, anti-pan-cytokeratin antibody; ISET, isolation by size of epithelial tumor cells; 7AAD, 7-aminoactinomycin D; DAPI, 4,6-diamidino-2-phenylindole; TBV, total blood volume (\sim 5 L for the avg. adult).

Expected values from flow cytometry detection are reported for Elutriation & FACS.

Sensitivity is equivalent to recovery.

^aMilliliters of blood.

^bSpike ratio = number of CTCs per 10^6 peripheral blood mononuclear cells (~60 CTCs/mL blood). ^c1 mL of blood assumed to contain 7.1 ×x 10^6 leukocytes (30).

with a purity that is well below 1% requiring subsequent immunological capture for their isolation. Following elutriation, spiked tumor cells recovered in the last elutriation fraction were isolated immunologically by FACS or immunomagnetic bead adsorption. To feasibly sort 1 billion cells, the acquisition rate during FACS was increased to 50 times that used during flow cytometry detection giving a throughput of 10⁸ cells/h. Fluorescent microscopy and real-time PCR analysis of cells sorted by FACS confirmed that events contained in the FSC vs. SSC tumor cell region and classified as CFSE positive EpCAM positive and CD45 negative were indeed tumor cells effectively isolated with a purity of ~90%. However, the increased speed used during sorting decreased the recovery of spiked tumor cells to 20%, three to fourfold less than identified after elultriation by flow cytometry analysis. The high loss is likely a result of increased coincidence at the higher sorting speeds (35,36), and as a result, an additional 15% of spiked tumor cells were cocaptured with leukocytes in the CD45 positive group. Repeat sorting of the CD45 positive group would be a simple way to moderately increase the recovery of the system. However, two-step sorting (a high-speed enrichment followed by a reduced rate sort with anticoincidence measures enabled) may provide a more efficient means to increase sensitivity without compromising specificity or increasing the required sort time (36). Immunomagnetic bead adsorption resulted in a lower recovery and a lower purity with greater variability than FACS when EpCAM was used alone to isolate spiked tumor cells from the last elutriation fraction (11). This may be attributed to the simultaneous elimination of CD45 leukocytes during FACS, and the additional elution step required by substrate based techniques that may also necessitate harsh treatment depending on the coupling configuration used. Microfluidic chambers containing antibody coupled microstructures offer an alternative means to immunologically capture CTCs. Activated with anti-EpCAM, such devices have achieved a tumor cell recovery of 61% with a purity of 50% in

preclinical spiking experiments, and have reported a greater CTC yield from blood samples obtained from metastatic cancer patients than immunomagnetic bead platforms (10). The improved performance has been attributed to the use of a steady laminar flow rate; however, this rate cannot exceed 1 mL of blood per hour per trial without negatively affecting sensitivity. The micrometer substrate geometry additionally rigid restricts the flexibility to simultaneously select cells using both positive and negative markers and physical character, a feature which is unique to FACS. As a consequence of the heterogeneity of metastatic disease and the lack of a specific CTC marker, a combination of multiple parameters is anticipated to improve the sensitivity and specificity of their enrichment and isolation (46,52-54).

There are few systems capable to process a large blood volume, and all are associated with increased cost and time, and necessitate the use of multiple steps to collect, enrich, and isolate CTCs decreasing the overall sensitivity of the process (55). Leukapheresis is the most common technique used to collect cells from a large blood volume. It represents a minimally invasive procedure requiring an experienced clinic, and can only be used for patients in relatively good standing with no contradictions to the procedure including organ dysfunction, active infection, immune deficiency, and active autoimmune disease (25). Following leukapheresis, CTCs may be enriched and isolated with any combination of techniques which offers a high process flexibility according to the desired outcome and the competencies of the research lab. Elutriation and FACS are both semiautomated systems in need of little material handling minimizing cell loss and the inherent variability of the process. However, each can be operated only by trained personnel, and elutriation requires the use of specialized disposable tubing sets adding further process cost. In conclusion, the combined use of elutriation and multiparameter rare event FACS was equivalent to or outperformed current techniques with regard to the

enrichment, recovery, and purity of isolated tumor cells. When combined with leukapheresis, the described process has at least a 500-fold greater capacity, expected to increase CTC yield and incidence (Table 2). Tumor cells and leukocytes isolated by this three-step process were also found to be viable, and easily identified and distinguished using multiple cytometric and molecular techniques. Based upon the findings of this study, the next step will be to evaluate the three-step process with cancer patients in direct comparative studies with current leading enrichment methods. An increase in CTC yield and incidence would improve the diagnostic utility of CTCs facilitating the early detection of minimal residual disease and the real-time monitoring of therapeutic benefit. In combination with a high purity, the increased yield would allow a robust phenotypical and molecular analysis of isolated viable CTCs increasing the potential to characterize the metastatic process more fully, identify and investigate new therapeutic targets and novel CTC markers, and develop an in vitro metastatic disease model to assess treatment response preclinically and realize individualized therapy.

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LITERATURE CITED

- Horner M, Ries L, Krapcho M, Neyman N, Aminou R, Howlader N, Altekruse SF, editors. SEER Cancer Statistics Review, 1975–2006. Bethesda, MD: National Cancer Institute; 2009.
- GLOBOCAN 2002. Cancer Incidence, Mortality and Prevalence Worldwide, Version 2. Lyon: IARCPress; 2004.
- Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519– 2529.
- Sasaroli D, Coukos G, Scholler N. Beyond CA125: The coming of age of ovarian cancer biomarkers. Are we there yet? Biomark Med 2009;3:275-288.
- du Bois A, Luck HJ, Meier W, Adams HP, Mobus V, Costa S, Bauknecht T, Richter B, Warm M, Schroder W, Olbricht S, Nitz U, Jackisch C, Emons G, Wagner U, Kuhn W, Pfisterer J. A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. J Natl Cancer Inst 2003;95: 1320–1329.
- Sood AK, Sorosky JI, Dolan M, Anderson B, Buller RE. Distant metastases in ovarian cancer: Association with p53 mutations. Clin Cancer Res 1999;5:2485-2490.
- Braun S, Schindlbeck C, Hepp F, Janni W, Kentenich C, Riethmuller G, Pantel K. Occult tumor cells in bone marrow of patients with locoregionally restricted ovarian cancer predict early distant metastatic relapse. J Clin Oncol 2001;19:368–375.
- Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LW, Uhr JW. Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci USA 1998;95: 4589-4594.
- Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Janicke F, Pantel K. Circulating tumor cells in breast cancer: Correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res 2005;11: 3678–3685.
- Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA, Toner M. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235–1239.

- Fan T, Zhao Q, Chen JJ, Chen WT, Pearl ML. Clinical significance of circulating tumor cells detected by an invasion assay in peripheral blood of patients with ovarian cancer. Gynecol Oncol 2009;112:185–191.
- Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW, Terstappen LW. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004;10: 6897-6904.
- Franklin WA, Glaspy J, Pflaumer SM, Jones RB, Hami L, Martinez C, Murphy JR, Shpall EJ. Incidence of tumor-cell contamination in leukapheresis products of breast cancer patients mobilized with stem cell factor and granulocyte colony-stimulating factor (G-CSF) or with G-CSF alone. Blood 1999;94:340–347.
- Gertler R, Rosenberg R, Fuehrer K, Dahm M, Nekarda H, Siewert JR. Detection of circulating tumor cells in blood using an optimized density gradient centrifugation. Recent Results Cancer Res 2003;162:149-155.
- 15. Baker MK, Mikhitarian K, Osta W, Callahan K, Hoda R, Brescia F, Kneuper-Hall R, Mitas M, Cole DJ, Gillanders WE. Molecular detection of breast cancer cells in the peripheral blood of advanced-stage breast cancer patients using multimarker real-time reverse transcription-polymerase chain reaction and a novel porous barrier density gradient centrifugation technology. Clin Cancer Res 2003;9:4865-4871.
- Zabaglo L, Ormerod MG, Parton M, Ring A, Smith IE, Dowsett M. Cell filtration-laser scanning cytometry for the characterisation of circulating breast cancer cells. Cytometry A 2003;55A:102-108.
- Zheng S, Lin H, Liu JQ, Balic M, Datar R, Cote RJ, Tai YC. Membrane microfilter device for selective capture, electrolysis and genomic analysis of human circulating tumor cells. J Chromatogr A 2007;1162:154-161.
- 18. Vona G, Estepa L, Beroud C, Damotte D, Capron F, Nalpas B, Mineur A, Franco D, Lacour B, Pol S, Brechot C, Paterlini-Brechot P. Impact of cytomorphological detection of circulating tumor cells in patients with liver cancer. Hepatology 2004;39:792–797.
- Vona G, Sabile A, Louha M, Sitruk V, Romana S, Schutze K, Capron F, Franco D, Pazzagli M, Vekemans M, Lacour B, Brechot C, Paterlini-Brechot P. Isolation by size of epithelial tumor cells: A new method for the immunomorphological and molecular characterization of circulatingtumor cells. Am J Pathol 2000;156:57–63.
- Sieuwerts AM, Kraan J, Bolt-de VJ, van der Spoel P, Mostert B, Martens JW, Gratama JW, Sleijfer S, Foekens JA. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. Breast Cancer Res Treat 2009;118:455–468.
- Judson PL, Geller MA, Bliss RL, Boente MP, Downs LS Jr, Argenta PA, Carson LF. Preoperative detection of peripherally circulating cancer cells and its prognostic significance in ovarian cancer. Gynecol Oncol 2003;91:389–394.
- 22. Wimberger P, Heubner M, Lindhofer H, Jager M, Kimmig R, Kasimir-Bauer S. Influence of catumaxomab on tumor cells in bone marrow and blood in ovarian cancer. Anticancer Res 2009;29:1787-1791.
- Friedlander ML. Prognostic factors in ovarian cancer. Semin Oncol 1998;25:305-314.
- 24. Chou T, Sano M, Ogura M, Morishima Y, Itagaki H, Tokuda Y. Isolation and transplantation of highly purified autologous peripheral CD34+progenitor cells: Purging efficacy, hematopoietic reconstitution following high dose chemotherapy in patients with breast cancer: Results of a feasibility study in Japan. Breast Cancer 2005;12:178–188.
- 25. Mazzolini G, Alfaro C, Sangro B, Feijoo E, Ruiz J, Benito A, Tirapu I, Arina A, Sola J, Herraiz M, et al. Intratumoral injection of dendritic cells engineered to secrete interleukin-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas. J Clin Oncol 2005;23:999–1010.
- 26. Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss TJ, Ciobanu N, Tallman MS, Kennedy MJ, Davidson NE, Sweet D. Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. Blood 1993;82:2605–2610.
- Ferrucci PF, Rabascio C, Gigli F, Corsini C, Giordano G, Bertolini F, Martinelli G. A new comprehensive gene expression panel to study tumor micrometastasis in patients with high-risk breast cancer. Int J Oncol 2007;30:955–962.
- 28. Bertolini F, Lanza A, Peccatori F, Zibera C, Gibelli N, Perotti C, Da Prada GA, Torretta L, Cocorocchio E, Martinelli G, Robustelli della Cuna G. Hematopoietic progenitor cell collection and neoplastic cell contamination in breast cancer patients receiving chemotherapy plus granulocyte-colony stimulating factor (G-CSF) or G-CSF alone for mobilization. Ann Oncol 1998;9:13–916.

- Shapiro HM, Schildkraut ER, Curbelo R, Laird CW, Turner B, Hirschfeld T. Combined blood cell counting and classification with fluorochrome stains and flow instrumentation. J Histochem Cytochem 1976;24:396–401.
- Lentner C. Geigy Scientific Tables. Basel, Switzerland: Ciba-Geigy Limited; 1984.
- Berger TG, Strasser E, Smith R, Carste C, Schuler-Thurner B, Kaempgen E, Schuler G. Efficient elutriation of monocytes within a closed system (Elutra) for clinical-scale generation of dendritic cells. J Immunol Methods 2005;298:61–72.
- 32. Dohnal AM, Graffi S, Witt V, Eichstill C, Wagner D, Ul-Haq S, Wimmer D, Felzmann T. Comparative evaluation of techniques for the manufacturing of dendritic cell-based cancer vaccines. J Cell Mol Med 2009;13:125-135.
- Pruitt JC, Hilberg AW, Morehead RP, Mengoli HF. Quantitative study of malignant cells in local and peripheral circulating blood. Surg Gynecol Obstet 1962;114:179–188.
- 34. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, Beitsch PD, Leitch M, Hoover S, Euhus D, et al. Circulating tumor cells in patients with breast cancer dormancy. Clin Cancer Res 2004;10: 8152-8162.
- Daugherty PS, Iverson BL, Georgiou G. Flow cytometric screening of cell-based libraries. J Immunol Methods 2000;243:211-227.
 Leary JF. Strategies for rare cell detection and isolation. Methods
- 36. Leary JE Strategies for rare cell detection and isolation. Methods Cell Biol 1994;42 (Part B):331-358.
- Coligan J. Immunofluorescence and Cell Sorting: Current Protocols in Immunology. New York, NY: Wiley; 1994.
 Donnenberg VS, O'Connell PJ, Logar AJ, Zeevi A, Thomson AW,
- Donnenberg VS, O'Connell PJ, Logar AJ, Zeevi A, Thomson AW, Donnenberg AD. Rare-event analysis of circulating human dendritic cell subsets and their presumptive mouse counterparts. Transplantation 2001;72:1946–1951.
- Donnenberg VS, Landreneau RJ, Donnenberg AD. Tumorigenic stem and progenitor cells: Implications for the therapeutic index of anticancer agents. J Control Release 2007;122:385–391.
- 40. Elshal M, Abdelaziz A, Abbas A, Mahmoud K, Fathy H, El Mongy S, El-Basyuoni S, Ahmed H, McCoy P. Quantification of circulating endothelial cells in peripheral blood of systemic lupus erythematosus patients: A simple and reproducible method of assessing endothelial injury and repair. Nephrol Dial Transplant 2009;24:1495–1499.
- Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. Clin Chem 2005;51:1598–1604.
- 42. Balic M, Dandachi N, Hofmann G, Samonigg H, Loibner H, Obwaller A, van der Kooi A, Tibbe AG, Doyle GV, Terstappen LW, Bauernhofer T. Comparison of two methods for enumerating circulating tumor cells in carcinoma patients. Cytometry B Clin Cytom 2005;68B:25–30.

- 43. Smirnov DA, Zweitzig DR, Foulk BW, Miller MC, Doyle GV, Pienta KJ, Meropol NJ, Weiner LM, Cohen SJ, Moreno JG, et al. Global gene expression profiling of circulating tumor cells. Cancer Res 2005;65:4993-4997.
- 44. Mostert B, Sleijfer S, Foekens JA, Gratama JW. Circulating tumor cells (CTCs): Detection methods and their clinical relevance in breast cancer. Cancer Treat Rev 2009;35:463–474.
- Paterlini-Brechot P, Benali NL. Circulating tumor cells (CTC) detection: Clinical impact and future directions. Cancer Lett 2007;253:180-204.
- Allan AL, Keeney M. Circulating tumor cell analysis: Technical and statistical considerations for application to the clinic. J Oncol 2010;2010:426218.
- 47. Hu Y, Fan L, Zheng J, Cui R, Liu W, He Y, Li X, Huang S. Detection of circulating tumor cells in breast cancer patients utilizing multiparameter flow cytometry and assessment of the prognosis of patients in different CTCs levels. Cytometry A 2010;77A:213–219.
- 48. Jung R, Kruger W, Hosch S, Holweg M, Kroger N, Gutensohn K, Wagener C, Neumaier M, Zander AR. Specificity of reverse transcriptase polymerase chain reaction assays designed for the detection of circulating cancer cells is influenced by cytokines in vivo and in vitro. Br J Cancer 1998;78:1194–1198.
- Jung R, Petersen K, Kruger W, Wolf M, Wagener C, Zander A, Neumaier M. Detection of micrometastasis by cytokeratin 20 RT-PCR is limited due to stable background transcription in granulocytes. Br J Cancer 1999;81:870-873.
- 50. Dandachi N, Balic M, Stanzer S, Halm M, Resel M, Hinterleitner TA, Samonigg H, Bauernhofer T. Critical evaluation of real-time reverse transcriptase-polymerase chain reaction for the quantitative detection of cytokeratin 20 mRNA in colorectal cancer patients. J Mol Diagn 2005;7:631-637.
- 51. Rosenberg R, Gertler R, Friederichs J, Fuehrer K, Dahm M, Phelps R, Thorban S, Nekarda H, Siewert JR. Comparison of two density gradient centrifugation systems for the enrichment of disseminated tumor cells in blood. Cytometry 2002;49:150–158.
- Zieglschmid V, Hollmann C, Gutierrez B, Albert W, Strothoff D, Gross E, Bocher O. Combination of immunomagnetic enrichment with multiplex RTPCR analysis for the detection of disseminated tumor cells. Anticancer Res 2005;25: 1803–1810.
- 53. Hager G, Cacsire-Castillo TD, Schiebel I, Rezniczek GA, Watrowski R, Speiser P, Zeillinger R. The use of a panel of monoclonal antibodies to enrich circulating breast cancer cells facilitates their detection. Gynecol Oncol 2005;98:211-216.
- 54. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer 2008;8:329–340.
- Goeminne JC, Guillaume T, Symann M. Pitfalls in the detection of disseminated non-hematological tumor cells. Ann Oncol 2000;11:785-792.