Confocal images of circulating tumor cells obtained using a methodology and technology that removes normal cells

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Abstract

A completely negative enrichment technology was used to detect circulating tumor cells, CTCs, in the peripheral blood of head and neck cancer patients. Of 32 blood samples, 63 percent contained CTCs and the number of CTCs identified per ml of blood collected ranged from 0 to 214. The final purity ranged from 1 CTC in 9 total cells to 1 CTC in 20,000 total cells, the final purity being both a function of the number of CTCs and the performance of the specific enrichment. Consistent with previous reports, CTC were positively identified if: 1) they contained a nucleus based on DAPI stain, 2) stained positive for cytokeratins, and 3) have a high nuclei to cytoplasmic ratio. In addition, for a blood sample to be considered positive for CTCs, the enriched sample must be positive for Epithelial Growth Factor Receptor, EGFR, as measured by RT-PCR. While most of the blood samples were obtained during surgery, a number were taken prior to, and during surgery. In all of the pre- and post-surgery paired samples, significant numbers of CTCs were detected. A number of these enriched samples were observed under confocal microscope in addition to the microscopic observations under traditional wide-field fluorescent microscope. As expected, the FITC stained cytokeratins appeared in the cytoplasm and the average size of these positively stained cells, on the cytospin, was in the range of 8-12 microns. Future studies will involve the investigation if cancer stem cell and mesenchymal markers are present on these CTCs and correlations of patient outcome to the number and type of CTC present.

Keywords

Circulating tumor cells; Immunomagnetic cell separation; Immunocytochemistry; RT-PCR; Squamous cell carcinoma of the head and neck (HNSCC)

Introduction

The genesis of overt metastases in a number of different types of cancer, including squamous cell carcinoma of the head and neck, HNSCC, is based on the concept that tumor cells that dissociate from the primary cancer obtain access to circulation either directly through blood

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vessels or after transit in lymphatic channels. According to the classical view of metastatic spread, the dissemination of cancer cells into the circulation occurs during the later stages of progression toward malignancy. However, recent research in breast cancer has demonstrated that this process in fact occurs early during tumor development. Thus, detection of such cells in patients with newly diagnosed solid tumors has been considered an appealing strategy to provide evidence of future metastasis [1-3]. Overall, the existence of circulating tumor cells (CTCs) and the settlement of these cells in secondary organs, such as liver, bone and lungs, as disseminated metastatic tumor cells (DTCs) is generally accepted [2]. Genome and transcriptome analyses of single disseminated tumor cells demonstrated that the majority of DTCs are cells with genetic aberrations compatible with malignancy. These cells are most likely direct descendants of the primary tumor, although the genetic changes can be incongruent with the dominant genotype of the corresponding primary tumor [4-6].

The detection of the presence of tumor cells outside the primary tumor would serve three purposes that could be potentially, clinically useful: 1) as unambiguous evidence for an early occult spread of tumor cells; 2) as a relevant risk factor for subsequent metastasis and, thus, poor prognosis; and 3) as a marker for monitoring treatment susceptibility. From a research perspective, genotyping and phenotyping of CTCs can provide a detailed insight into the metastatic process and permit direct exploration of targeted treatment strategies [2,3].

The available literature regarding the prognostic relevance of the presence of DTC in bone marrow was inconsistent for breast cancer until a recent pooled analysis [7]. The results of this large study with over 4000 patients showed that the presence of DTCs in bone marrow of patients with stage I-III breast cancer is a strong, adverse prognostic factor for all the clinically relevant end-points. The patient sample included disease-free, distant disease-free, breast cancer specific and overall survival in the entire cohort. It also included subsets of patients treated with chemotherapy only, endocrine therapy only, and in those patients with small primary tumors and no lymph node metastases who received no systemic adjuvant therapy. A different study with metastatic breast cancer patients indicated that the detection of greater than 5 CTCs per 7.5 mls of peripheral blood adversely affects progression-free and overall survival and is a predictive marker for lack of response to treatment [8]. This correlation was developed through studies performed in the first FDA approved diagnostic device to detect CTC; the CellSearch™ system, currently sold by Veridex, LLC. This system is a semi-automated immunomagnetic enrichment system that involves positive selection of CTCs using anti-EpCAM antibodies coated with ferrofluids. The enriched CTCs are then labeled for immunofluorescent imaging with DAPI, anti-CD45 antibodies to label blood cells and anti-cytokeratin antibodies to label CTCs. The CellSearch system has been used in a number of other studies, including a much larger study where 70% of over 1400 metastatic breast cancer patients had at least 5 CTCs per 7.5 ml of blood [9].

Several studies suggest that CTCs are descendents of the corresponding primary tumor [10-13]. In contrast, it is thought that DTCs do not share such broad molecular similarities; therefore, it remains unanswered if a clinically desirable blood test for CTC detection would be able to provide the same information as the bone marrow test for DTC detection.

From a clinical point of view it will be necessary to determine what percentage of CTCs and DTCs are detectable, but non-viable, and which of them are actually capable of forming solid metastases Several studies show the ability to profile DTCs and to differentiate their biological properties using anti-cytokeratin antibodies in combination with antibodies against tumor-associated markers [14-19]. Differential gene or protein expression studies show that CTCs have malignant potential [10,13]. In addition to tumor cell profiles, information on markers such as Epidermal Growth Factor Receptor (EGFR) [20], Epithelial Cell Adhesion Molecule (EpCAM) [17], uPA receptor (uPAR) [15], Extracellular matrix metalloproteinase inducer

(EMMPRIN) [14] may have therapeutic implications for targeted therapy strategies. Consequently, more efficient and cost effective methods to enrich CTCs and DTCs could potentially expedite and support further important studies on tumor cell characterization and by doing so, potentially improve the prognostic and predictive value of CTCs and DTCs.

In contrast to breast cancer, few studies on CTCs in HNSCC have been conducted. One these studies was by Partridge et al. (2003) [21] that reported that presence of CTC in samples of bone marrow and central venous blood, collected preoperatively or post-operatively from head and neck cancer patients, indicated a high risk of local and distant recurrence and reduced survival.

CTC detection technology

Several methodologies have been developed to detect and characterize DTCs in bone marrow or CTCs in peripheral blood of cancer patients. These methodologies fall under two main categories: Immunological based assays and molecular based assays. Although these approaches can be used directly, an enrichment step prior to the detection is preferred. A number of methodologies exist for enriching rare cancer cells, including density gradient separation to enrich for nucleated cells (i.e. Ficoll), and magnetic cell separation either targeting the cancer cell, positive immunomagnetic cell separation, PIMS, or negative immunomagnetic cell separation, NIMS, where normal blood cells are targeted. Unfortunately, most of the reported studies using some form of magnetic cell separation to separate or enrich rare cancer cells do not provide data, or complete data, on the performance of the magnetic separation step. Consequently, it makes comparison of the performance of technology, as well as the overall study difficult or impossible.

Recently, two new approaches to enrich for CTC's have been reported. In one of these approaches, a microfluidic device containing anti-EpCAM antibodies conjugated to "microposts" was developed to facilitate interactions between CTCs and the posts, when whole blood flows through the device under controlled laminar flow conditions. It is reported to have high specificity and sensitivity; however, since it is another version of positive selection, it suffers from the bias of assuming that all CTCs express the epithelial surface marker EpCAM [22]. The other method involves enrichment of CTCs by filtration before genomic analysis. Basically, this approach assumes that the CTC are bigger than typical blood cells; therefore, the normal cells will flow through the filtration media while the CTC are retained [23]. As with other positive selection approaches, this has an intrinsic bias that CTC are different in size than normal blood cells.

In contrast, the Chalmers and Zborowski labs have developed a purely negative enrichment technology in which only normal blood cells are targeted and removed, thereby allowing rare, non-hematopoietic cells to be significantly enriched (Yang et al. 2009) [24]. They reported that this system is able to reduce the number of normal blood cells in a cancer patient's blood from 4.05×10^9 to 8.04×10^3 cells per ml and still recover, on average, 2.32 CTCs per ml of blood. For all of the cancer patient blood samples tested at the time of publication, and in which CTCs were detected (20 out of 26 patients) the average recovery of CTCs was 21.7 per ml of blood, with a range of 282 to 0.53 CTC.

Materials and Methods

Blood collection

10 to 18.5 ml peripheral blood was obtained from patients who presented with, and underwent surgical resection for HNSCC and that have not been previously treated for this disease. Blood samples were collected in green-top BD-Vacutainer (Cat#367874, BD Biosciences) and stored

at 4°C until processing. Samples were processed within 24 hrs after procurement. Operators were blinded to clinical correlative information during the cell suspension processing and analysis.

Cell culture

The head and neck cancer cell line SCC-4 was purchased from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Cat#30-2002, ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Cat#30-2020, ATCC, Manassas, VA) and 1% Penicillin-Streptomycin (Cat#30-2300,ATCC, Manassas, VA). Cells were harvested using Accutase™ (Cat#AT104, Innovative Cell Technologies, Carlsbad, CA) as per manufacturer's instruction.

Reagents used

Tetrameric antibody complexes, TAC, from Stem Cell technologies (Vancouver, BC) were used to immunomagnetically label peripheral blood lymphocytes, PBLs. The specific TAC used in this study targeted the CD45 cell surface antigen and the dextran coated, magnetic nanoparticles. 0.5ul of the TAC complex was added per million cells and the cell suspension was incubated for 30 min at room temperature in a shaker. Without washing the cell suspension, 1 ul of the magnetic nano particle suspension per million cells was then added and incubated for 15 min at room temperature. The cells are then washed with labeling buffer, centrifuged and resuspended in labeling buffer.

Separation Methodology

The immunomagnetic separation was carried out as described in Yang et al 2009[24] and will only be summarized here. An overall view of the separation process is shown in Figure 1. Red blood cells in the blood samples were lysed by mixing blood and lysis buffer (154mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) at a ratio of 1:25, incubating it for 5 min at room temperature before pelleting the remaining blood cells at 350xg for 5 min. Nucleated cell concentration was estimated by diluting 20 μl with 3% acetic acid (1:25) and counting the cells using a Hemocytometer. The cell suspension obtained, consisting mostly of nucleated cells, is then first labeled with FcR blocking reagent and then anti-CD45 TAC and incubated for 30 min at room temperature on a shaker. Without washing the cells, magnetic nanoparticles are then added to the cell suspension and incubated for 15 min at room temperature in a shaker. The immunomagnetically labeled cell suspension is subsequently run through the deposition Quadrupole Magnetic Sorter (dQMS) system to obtain an enriched sample.

Confocal images and immunostaining

Confocal images were obtained using a Zeiss LSM 510 confocal/multi photon laser scanning microscope equipped with Argon/2 laser (458, 477, 488, 514 nm) and a Titanium Sapphire laser (750 nm). The cells were viewed with a 63X (NA 1.2) apochromatic water objective and images of different fields were taken. The microscope was set up to take multi track images and the excitation and emission filter sets configured individually so that there is no fluorescence bleed-through between the channels. The argon (488 nm) laser with appropriate, emission filters was used for the visualization of FITC. The DAPI staining was viewed by the use of a tunable IR laser which excites the fluorophore using two photons at the point of focus. An aliquot of the enriched sample after immnunomagnetic separation was preserved in neutral buffered formalin prior to preparing a cytsopin. After washing the slides with PBS (Cat#SH3025601, Thermo Sci Hyclone), the cells were incubated with anti-cytokeratin FITC CK3-6H5 (1:10) (Cat#130-080-101, Miltenyi Biotech) for 30 min at 37°C followed by a 30 min blocking with PBS pH 7.4 with1%BSA at room temperature. The slides were then washed

with PBS for 15 min air dried, and mounted with Vectashield® mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Cat# H-1200, Vector Laboratories).

RT-PCR analysis

As presented previously by Tong et al. (2007) [25], and Yang et al. (2009) [24], a portion of the sample before enrichment and after enrichment was preserved in RNA*later* for further molecular analysis. RNA was extracted from these samples using Trizol reagent (Cat# 15596-018) and/or Picopure RNA isolation kit (Cat#KIT0204, Molecular Devices) depending upon the number of cells in the sample. Aliquots of 1μg RNA was reverse transcribed according to manufacturer's protocol. Following reverse transcription 2μl of the cDNA product was amplified for EGFR generating a band at 301bp. The EGFR primer sequences are as follows: GGGAGCAGCGATGCGA and CTCCACTGTGTTGAGGGCAAT. 35 cycles of PCR were performed, each consisting of a denaturation step (94°C for 1min), an annealing step (60°C for 1min) and an extension step (72°C for 1.5 min). Final extension for 10min at 72°C was allowed and the mixture was cooled down to 4°C. RT-PCR products were separated by electrophoresis on a 1.5% agarose gel containing Ethidium Bromide and analyzed by direct visualization, and photographed, under UV light. Figure 2 presents representative photographs of a positive product from a patient, a negative product, and a lane containing molecular weight bands.

Results

As reported previously by Yang et al. (2009) [24], for a cell to be considered a CTC by visual observation using a fluorescent microscope, it must: 1) be positive for a nucleus based on DAPI stain, 2) be positive for cytokeratins based on FITC fluorescence, and 3) have a high nuclei to cytoplasmic ratio. In addition, for a blood sample to be considered positive for CTCs, the enriched sample must be positive for EGFR, based on visual observations of gels run on RT-PCR product. For a sample size of 32 blood samples from different patients, 63 percent of the samples contained CTCs, based on the identification protocol defined above. The number of CTCs detected per ml of blood collected ranged from 0 to 214. The final number of CTCs were estimated by counting the number of CTCs on the slide and multiplying it by the corresponding dilution factor. Figure **3** is a histogram indicating the distribution of these 32 samples with respect to the number of CTCs per ml of blood. Using the optimized enrichment protocol reported by Yang et al. (2009) [24], the final purity of the enriched sample is a function of the number of CTCs recovered as a well as the final number of non-CTC's remaining. In our study, this purity defined as the ratio of number of CTCs to number of non-CTCs ranged from 1 CTC in 9 total cells to 1 CTC in 25,000 total cells. All of these patient samples were also analyzed for EGFR expression by RT-PCR. For samples in which no cytokeratin positive cells were identified based on visual inspection, the RT-PCR analysis for EGFR was also negative. Conversely, for all but two of the cytospins that were considered positive for CTCs, the samples were positive for EGFR.

To further determine the accuracy of our ability to visually identify and characterize the CTCs, we analyzed a number of the cytospins of the enriched samples using confocal microscopy. Figure 4 is a set of confocal images of an enriched, peripheral blood sample from a HNSCC patient. A total of 14 "z slices" were taken, and Figures 4A through 4D are representative, progressive slices moving from the bottom of the cytospin to the top. As can be observed, the nuclei are most pronounced in Figures 4B and 4C, while the cytokeratin is present around the nuclei, as expected. Also, it can be noted that some of the cells in the field of view do not fit the criteria to be considered a CTC. In particular, in the lower left is a cell, negative for cytokeratins and with a "kidney shaped" nucleus, suggestive of being a monocyte or granulocyte. Near the center of Figures 4A-4D, cytokeratin positive cellular debris can be observed. While not counted as a CTC, this debris most likely represents a CTC that had

undergone apoptosis. For the specific patient sample shown in Figure 4, a total of 214 CTCs per ml of blood was determined, and the final purity was 1 CTC in 31 non-cytokeratin positive cells. Obviously, visual inspection of Figure 4 indicates a different ratio of cytokeratin positive to cytokeratin negative cells. Care was taken in the inspection of the cytospin to find this specific image to maximize the number of cytokeratin positive cells in a single field of view.

As a control, a cytospin using the same staining protocol used on patient samples was made using cultured SCC-4 cells, and a confocal image of a single, z-slice of these stained cells is presented in Figure 5. As expected, the cells stained positively for the nuclei and cytokeratins which surround the nuclei.

To demonstrate the reproducibility of these confocal images and the presence of CTCs in patient's blood prior to surgery, samples were taken before and after surgery and processed using the same separation and staining protocol mentioned earlier. Figures 6A and 6B are representative images of two cytopsin from a sample taken prior to, and during surgery, respectively.

Discussion

It is suggested that a number of significant conclusions can be made from the results presented in this study. First, a large range in concentration of CTCs in the peripheral blood of cancer patients exits. Second, confocal images indicate that the reagents used to target cytokeratins, a FITC conjugated pan anti-cytokeratin antibody, did in fact target structures in the cell's cytoplasm. Third, cytospins can be made that contain a very high percentage of cytokeratin positive cells. Fourth, these CTCs can be present in the blood of cancer patients, at high concentrations, prior to surgery, eliminating the possibility that the presence of CTCs is the result of tumor manipulation during surgery; however, it is still possible that manipulation can increase the number of CTCs. Fifth, CTCs can be of similar size to PBL's. It has been suggested in other publications that CTCs are typically larger than normal blood cells.

Ongoing studies in our laboratory involve studying the expression of other markers which have been reported to be potentially important with respect to metastatic cancer. These include cancer stem cell (CSC) markers which have been reported, or suggested to be identified, in various solid cancers including breast, prostate, colon, brain, pancreatic, and head and neck cancer [26-32].

Since a purely negative enrichment technology is used, it is also possible, especially with multicolor confocal microscopy, to investigate if any of the CTCs have mesenchymal characteristics. There is a growing body of literature which suggests that epithelial cancer undergoes a process called *epithelial-mesenchymal transition* (EMT) through which the tumor cells undergo a loss of polarity, lose cell-cell junctions, and acquire a mesenchymal phenotype. It is also proposed that this mesenchymal phenotype is "motile", "migratory" and "invasive" and it also promotes the escape of the cancer cells from the primary site and leads to the development of metastases [33-36].

Acknowledgments

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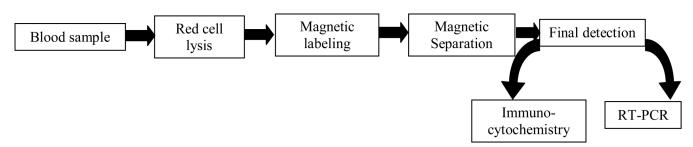
Figure 1.
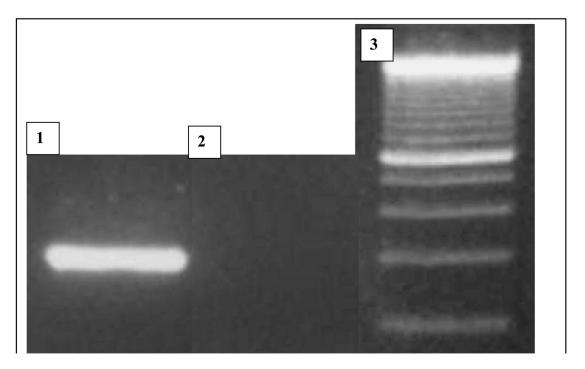Flow diagram of current process to enrich for rare cancer cells in human blood.

Figure 2.
Photograph of a gel of a RT_PCR product for a positive sample, lane 1, and negative sample, lane 2, and molecular weight bands, lane 3.
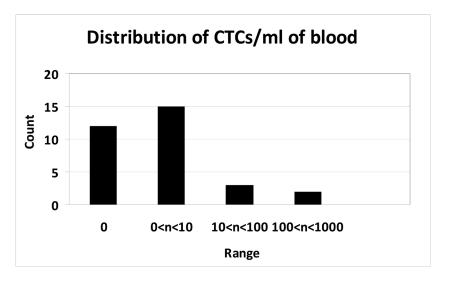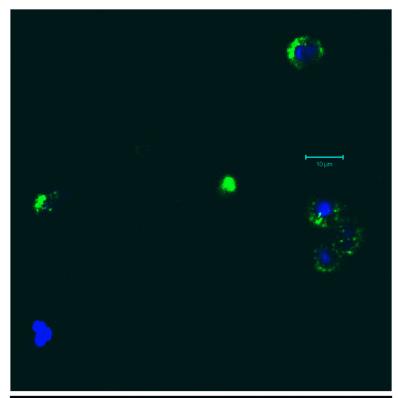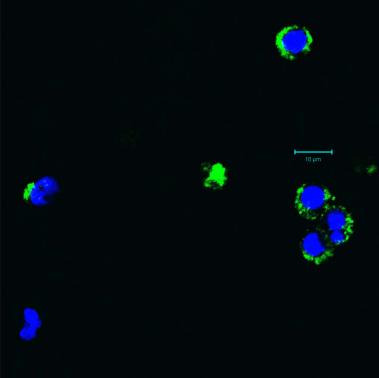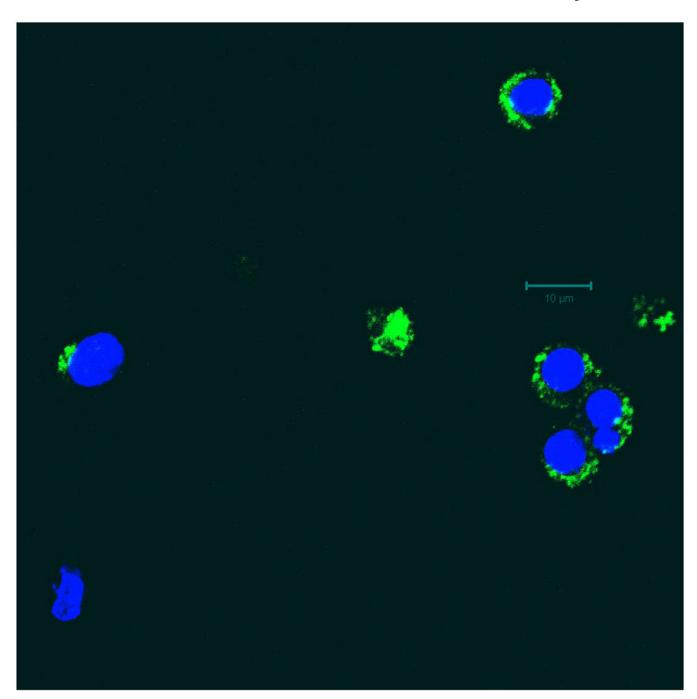
Figure 3.
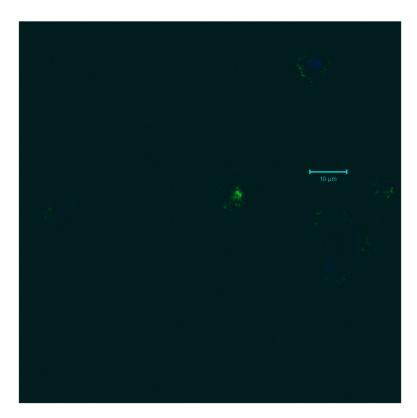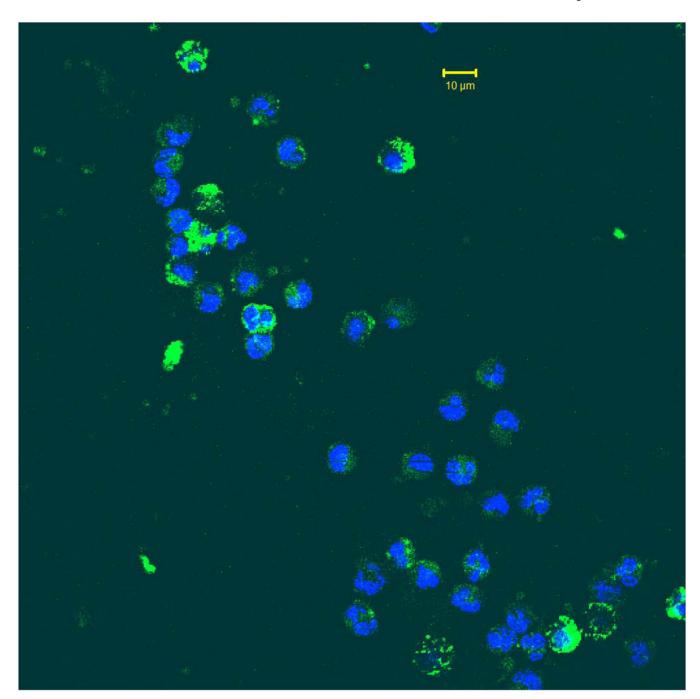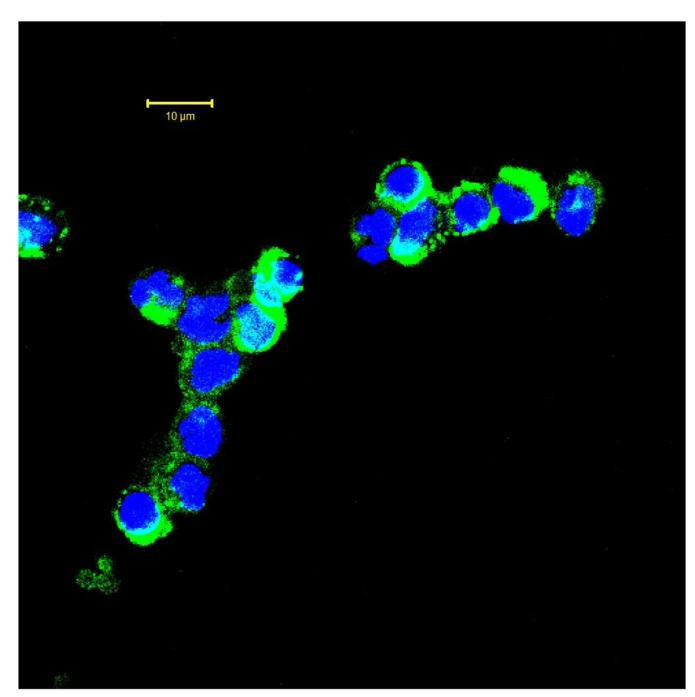Histogram of the number of CTC's detected per milliliter of patient blood sample (n=32).
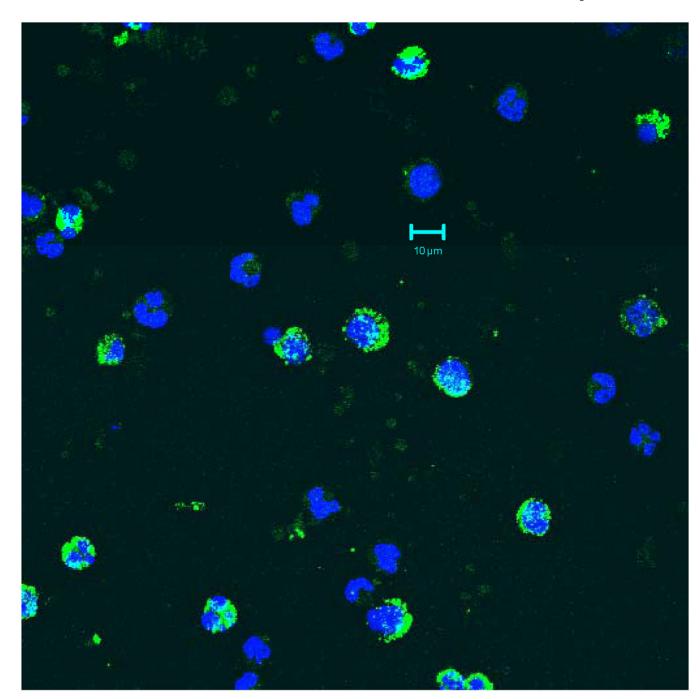
10 µm

Figure 4.
Set of progressive "z-slices" of confocal images of stained cells on a cytospin slide of an enriched peripheral blood sample from a head and neck cancer patient. Each of the four images is a computer combined digital image of separate images filtered for DAPI (nuclei) staining (blue) and FITC (cytokeratin) staining (green-yellow). A total of 14 "z-slices" were obtained and 4A, 4B, 4C, and 4D correspond to slices 3, 5, 6, and 9, respectively. Note, the lower the number, the further from the objective; consequently, the largest view of nuclei, and the surrounding cytokerain, is presented in 3B and 3C.

Figure 5.
A combined (all z slices) of a confocal image of SCC4 cells after being processed on a cytospin using the same labeling protocol as was used on the blood sample presented in Figure 4.
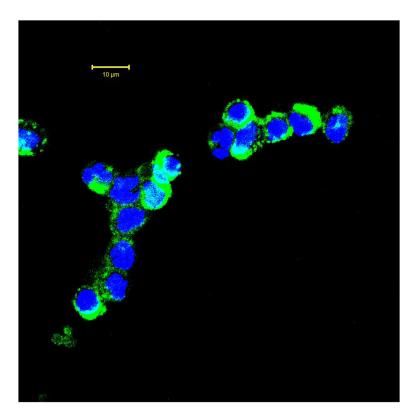
Figure 6.
A confocal image of a stained cytospin of an enriched peripheral blood sample prior to, 6A, and during surgery, 6B. Note the presence of what appears to be an emboli in 6B.