# Circulating Tumor Cell Detection and Characterization Using the Amnis ImageStream$^X$ System

David Basiji, William Ortyn, Cathleen Zimmerman, Vidya Venkatachalam, Thaddeus George, Raymond Kong
Amnis Corporation, 2505 Third Ave., Seattle, WA, 98121, USA

## Abstract

The number of circulating tumor cells (CTC) in peripheral circulation has been shown to be a predictor of survival in metastatic cancer. However, CTC enumeration is challenging to perform in a reliable and efficient manner despite the fact that CTCs are morphologically and phenotypically distinct from peripheral blood cells. The extremely low abundance of CTCs in the blood (~1 CTC per milliliter) makes enumeration subject to Poisson statistics, such that the variance in the number of CTCs detected from sample to sample is equal to the mean. As a result, high enrichment yield and high detection efficiency are essential to maximize both assay sensitivity and reproducibility.

Many existing techniques for the enumeration of CTCs rely on enrichment via antibodies against EpCAM. Because this enrichment strategy relies on a single parameter, its efficiency may be compromised if the patient's CTCs exhibit low EpCAM expression. The addition of other enrichment parameters (e.g. CD146 and/or other markers) can reduce the vulnerability of the assay to EpCAM expression variation but also increases the number of cells, CTC or otherwise, to analyze following enrichment.

Ultimately, the optimal degree of CTC enrichment is dictated by the speed of the analytical technique used for enumeration. As the speed of the analytical technique increases, enrichment strategies with greater sensitivity to CTCs but lower degrees of enrichment can be employed as long as the subsequent analysis can successfully discriminate CTCs from the remaining non-tumor cells. Flow cytometry is extremely fast but does not provide imagery of the cells necessary for the identification of false positives, which is critical when treatment decisions are being made on the basis of five or fewer cells. Conversely, most imaging platforms lack sufficient speed to analyze all the cells in the sample in a reasonable period of time. Ideally, the analytical platform employed would have both the imaging speed necessary to accommodate multiparametric enrichment as well as multiparametric analysis, thereby allowing the use of morphologic, phenotypic, and functional probes to both discriminate CTCs from non-tumor cells and to provide an assessment of tissue origin, malignancy, and other information of diagnostic and therapeutic value.

The ImageStream$^X$ imaging flow cytometer is being evaluated as an analytical platform for the enumeration and functional assessment of CTCs. The system is capable of producing 12 simultaneous high resolution images of each cell directly in fluid suspension and at rates exceeding 4000 cells/sec. The complement of cell imagery includes brightfield (transmitted light), darkfield (scattered light) and fluorescence from up to 10 probes. By combining the speed and fluorescence sensitivity of conventional flow cytometry with the information richness of microscopy, the ImageStream platform may enable the enumeration and functional assessment of CTCs without the need for high levels of enrichment.

Several CTC feasibility experiments were performed using the ImageStream$^X$ with the following goals:

1. Determine the degree to which brightfield and darkfield morphology may assist in the discrimination of leukocytes from CTCs.
2. Compare clinical CTC imagery produced by the ImageStream$^X$ system to imagery produced from the same patient sample by the Veridex CellTracks® system.
3. Evaluate STEMCELL Technology's RosetteSep® system as a pre-enrichment methodology for CTC analysis via the ImageStream$^X$ system.

## Background

The ImageStream$^X$ operates in a manner similar to a conventional flow cytometer. A cell suspension is pumped into a flow cell and is hydrodynamically focused into a thin core using sheath fluid. This process improves imaging by physically separating the cells from each other, positioning them at the plane of best focus, and surrounding them in clear fluid to produce a pristine image background.

As shown in Figure 1 below, the cells are illuminated from the side by up to five excitation lasers and from behind by an LED array. The LED trans-illuminator allows brightfield imaging while the laser illumination excites a wide variety of standard probes for fluorescence imaging. A dedicated near-infrared laser is used to illuminate the cells for darkfield imaging. The lasers are reflected back on to the cells to increase illumination uniformity and to nearly double the sensitivity of fluorescence detection.

Light is collected via one of three air-coupled imaging objectives (60X/0.9NA, 40X/0.75NA, 20X/0.7NA) mounted on a motorized linear stage. The stage allows precise motion in both the Z and X axes for automatic tracking of any core drift across the field of view or through focus. The optical system achieves diffraction-limited performance over a spectral range of 420 – 800 nm.

Prior to final image formation, the collected light passes through a spectral decomposition system placed in aperture space. The system consists of a fanned array of dichroic mirrors, each reflecting light of a specific spectral bandwidth while passing the remainder. The mirrors are mounted in a fixture that allows them to be steered at different angles such that they produce six distinct spectral images of each cell arrayed side-by-side on a CCD camera. This system allows the simultaneous imaging of multiple fluorescent probes per cell along with each cell's darkfield and brightfield images. The ImageStream$^X$ instrument can accommodate two spectral decomposition/camera assemblies to produce up to 12 images per cell in a single pass.

The custom 12-bit CCD camera(s) in the instrument operate in time-delay integration (TDI) mode, a method of electronically tracking cell motion by shifting the electronic image signals down the sensor at the same rate the projected cell images move due to fluid flow. The velocity of the cells is measured via an independent optical system and fed back to the TDI camera for readout synchronization. For a given flow speed, TDI operation increases the signal integration time by ~1000 fold, greatly increasing sensitivity. Using TDI, the ImageStream$^X$ achieves fluorescence sensitivity superior to conventional flow cytometers while producing ~50,000 cell images per second (~4,000 cells/sec X 12 images/cell).

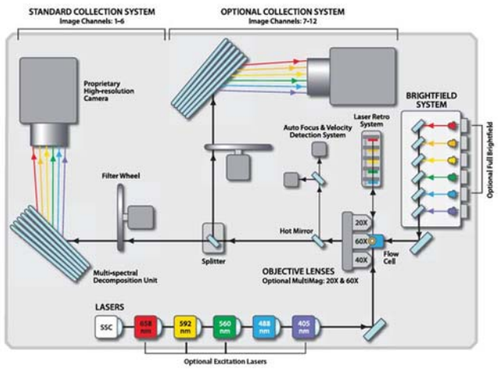


**Figure 1. ImageStream$^X$ Optical Diagram**

## Results

**1. Determine the degree to which brightfield and darkfield morphology may assist the discrimination of leukocytes from CTCs.**

In this experiment, performed in collaboration with Jaco Kraan and Jan Gratama of the Erasmus Medical Center (Rotterdam, NL), approximately 100 CAMA breast cancer cells were taken from culture and spiked into 7.5 ml of whole blood to model CTCs in a patient sample. The spiked sample was then enriched and probed with DAPI, anti-cytokeratin-PE, and anti-CD45-APC using the Veridex CellSearch® system. The Veridex cartridge was then sent to Amnis, where the contents were resuspended in 50 µl and imaged at 40X magnification on the ImageStream$^X$. Example CAMA cell and leukocyte images are shown in Figure 2.

Qualitatively, the brightfield and darkfield imagery readily distinguish CAMA cells from leukocytes based on the CAMA cells' increased size and granularity, which manifests as a bright and highly textured darkfield image. Quantitative image analysis reveals that the average CAMA cell brightfield area is four times that of the average residual leukocyte area (~400 µm$^2$ vs. ~100 µm$^2$) while the average total darkfield intensity of the CAMA cells is over ten times that of the residual leukocytes.

Nuclear area is currently used as a parameter to discriminate CTCs from leukocytes and the nuclear area of the CAMA cells is over 2.5-fold greater than that of the leukocytes (~180 µm$^2$ vs. ~70 µm$^2$). However, both the brightfield area and darkfield intensity each provide discrimination power at least as great as nuclear area and, when used in conjunction with existing parameters, may significantly improve the ability to discriminate CTCs from non-CTCs after enrichment.

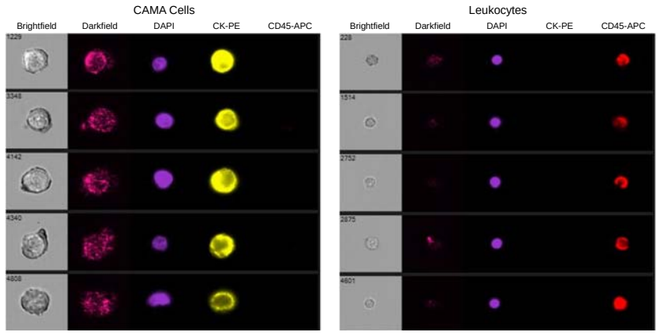


**Figure 2: ImageStream$^X$ imagery of CAMA cells and leukocytes following CellSearch® enrichment**

Imagery was acquired at 40X magnification and each cell is represented by a row of five images, indicated above each column. Brightfield illumination was via blue LED. Darkfield illumination was via 785 nm laser. DAPI excitation was via 405 nm laser illumination. PE excitation was via 488 nm laser excitation. APC excitation was via 658 nm laser illumination.

**2. Compare clinical CTC imagery produced by the ImageStreamX system to imagery produced by the Veridex CellTracks® system.**

In this experiment, a patient sample provided by Jaco Kraan and Jan Gratama of the Erasmus Medical Center (Rotterdam, NL) was enriched and probed with DAPI, anti-Her2-FITC, anti-cytokeratin-PE, and anti-CD45-APC using the Veridex CellSearch® system, followed by imaging on the CellTracks® system. The Veridex cartridge was then sent to Amnis, where the contents were resuspended in 50 µl and imaged at 40X magnification on the ImageStream$^X$. CTC imagery from both platforms is shown in Figure3.

The ImageStream$^X$ imagery compares favorably to that of the CellTracks® system. In addition, an analysis of the Her2-FITC imagery from both platforms demonstrates that the signal to noise ratio of the total Her2 signal from each cell is approximately 100-fold higher with the ImageStream$^X$ compared to the Veridex® system.

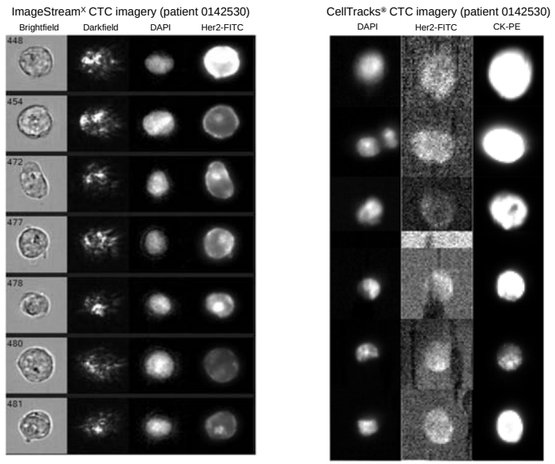


**Figure 3: ImageStream$^X$ and CellTracks® Imagery of the same CTC sample.**

**3. Evaluate STEMCELL Technology's RosetteSep® as a pre-enrichment methodology for CTC analysis via the ImageStream$^X$ system**

In this experiment, CAMA cells were spiked into 1 ml of fresh whole blood which was then processed using the RosetteSep® Human Circulating Epithelial Cell Enrichment Cocktail kit (cat. # 15127) from STEMCELL Technologies. Five different spiking levels were employed, from a maximum of 500,000 CAMA cells to a minimum of 50 cells with10-fold dilution steps in-between. The enrichment process is illustrated in Figure 4.

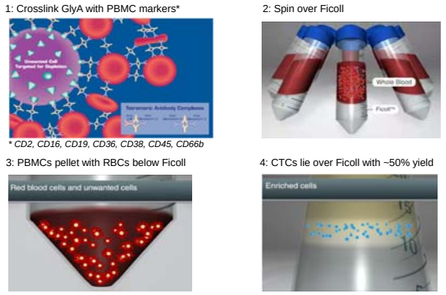


**Figure 4: RosetteSep® Human Circulating Epithelial Cell Enrichment Cocktail process**

After enrichment, the cells were labeled with an anti-CD45-PE marker as well as an anti-EpCAM-FITC marker, re-suspended in 50 µl, and imaged on the ImageStream$^X$ system using 100 mW of 488 nm laser excitation. Brightfield, darkfield, FITC fluorescence, and PE fluorescence images were generated simultaneously from each cell at 40X magnification.

The first step in data analysis utilized the information gleaned from prior experiments showing that the brightfield area and darkfield total intensity parameters both discriminated CAMA cells from leukocytes. The imaged data from the 500,000 cell spiked sample were plotted using these two parameters, as shown in Figure 5, revealing a well-defined population of cells having high brightfield area and high darkfield intensity. Visual inspection of the imagery linked to each dot on the plot verified that the cells in the population were CAMA cells, though their morphologic heterogeneity was high. Cells in the remaining populations were also inspected and verified to consist of debris, residual erythrocytes, and clusters of three to five cells.

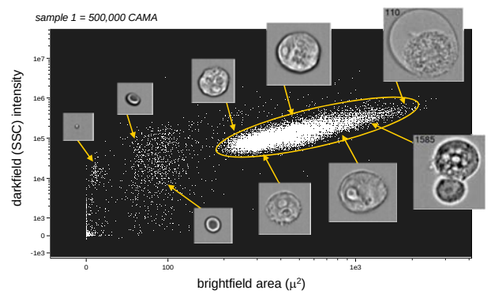


**Figure 5: Dot plot of brightfield area vs. darkfield intensity of post-enrichment cell sample**

Subsequent data analysis was performed on the putative CAMA population having high brightfield area and high darkfield intensity. Cells from this population were re-plotted using the parameters of EpCAM-FITC total intensity vs. CD45-PE total intensity, similar to what would be done in a standard flow cytometric analysis. Gates were drawn to define an EpCAM- CD45+ population of putative leukocytes as well as an EpCAM+ CD45- population of putative CAMA cells, plus an intermediate population of cells.

Visual inspection of the populations revealed that the EpCAM- CD45+ population consisted almost completely of leukocytes that were conjugated to one or more erythrocytes, accounting for their increased scatter intensity and brightfield area. The EpCAM+ CD45- population was a highly pure population of CAMA cells but occasional doublets could be found. The intermediate population in the EpCAM- CD45- region of the plot consisted mainly of erythrocyte conjugates. However, the cells in the EpCAM+ CD45+ region of the plot consisted of CAMA cells conjugated to a leukocyte or with an adherent patch of leukocyte membrane, presumably from a leukocyte detached during sample processing.

It is important to note that this analysis employed parameters that have direct equivalents in conventional flow cytometric systems. However, without the ability to visualize the cells, conventional flow cytometry is prone to false negative artifacts, both by mis-classifying EpCAM+ CD45+ events as leukocytes and by under-counting CTC doublets as single cells or rejecting them entirely from the analysis with doublet discrimination circuits.

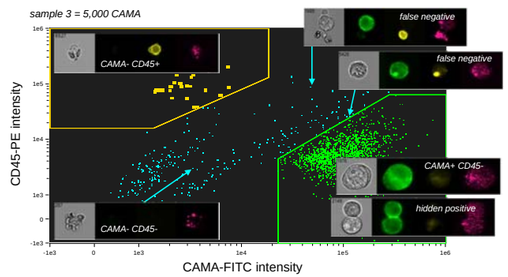


**Figure 6: Plot of EpCAM-FITC intensity vs. CD45-PE intensity of putative CAMA cell population defined in Figure 5**

Each of the samples was analyzed according to the previous description, including visual detection of potential false negative events, with the corresponding dot plots shown below for samples 2 – 5.

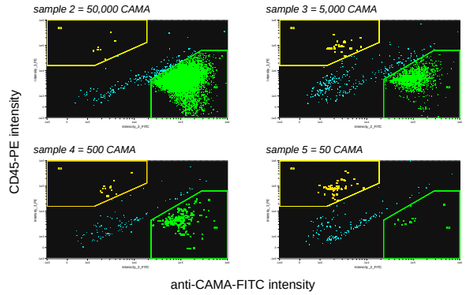


**Figure 7: Plot of EpCAM-FITC intensity vs. CD45-PE intensity for the samples spiked with 50,000 – 50 CAMA cells**

The number of detected CAMA cells for each sample are shown in Table 1. On average, approximately 30% of the spiked cells were detected in each sample, corresponding to a detection limit of about 3 CTCs per ml of blood.

The combined yield is the product of the enrichment yield and the sample utilization efficiency of the imaging system. The ImageStream$^X$ is currently optimized for high sample throughput, reflecting its primary role as a research instrument, and has a known sample utilization efficiency of approximately 50%. Based on this, it is estimated that the enrichment yield achieved in the initial spiking experiments described here was 50-60%.

The fluidic system of the ImageStreamX has been redesigned to achieve better than 95% sample utilization efficiency and subsequent optimization of the enrichment protocol has improved the enrichment yield to ~90%. The product of these yield factors combined with an increase in the working volume to 7.5 ml will result in a detection limit of approximately 1 CTC per 7 ml of blood.

| CAMA cells spiked | CAMA cells detected | Combined yield | Detection limit (CTC/ml) |
|---|---|---|---|
| 500,000 | 163,665* | 32.7%* | 3.0 |
| 50,000 | 12,483 | 25.0% | 4.0 |
| 5,000 | 1,781 | 35.6% | 2.8 |
| 500 | 316 | 63.2% | 1.6 |
| 50 | 15 | 29.1% | 3.4 |

**Table 1: Yield and detection limit for each spiked sample**

## Conclusions

High speed analytical platforms, including flow cytometry and rapid cell imagers, have the potential to improve CTC detection and analysis by allowing more sensitive / less specific enrichment strategies that are both high-yield and robust in the face of CTC biological variability. The studies described here demonstrate the benefits of the ImageStream$^X$ imaging flow cytometer for CTC imaging, including high image quality, a diversity of imaging modes, and high fluorescence sensitivity.

Relative to the Veridex CellTracks® imaging platform, the ImageStream$^X$ offers additional brightfield and darkfield imaging modes. The studies described herein demonstrate that these additional imaging modes provide both qualitative and quantitative morphologic information of use in discriminating CTCs from residual leukocytes and debris. In addition, the fluorescence sensitivity of the ImageStream$^X$ has been shown to be approximately 100-fold greater than the CellTracks® platform. This allows for the enhanced quantitation of phenotypic, RNA, and DNA probes, which can be multiplexed using up to 8 fluorescent dyes.

Though conventional flow cytometry provides both high analytical speed and high fluorescence sensitivity, the technique is prone to false negative artifacts in CTC enumeration. The cell imagery provided by the ImageStream$^X$ readily allows the detection of these false negative artifacts which cannot be tolerated in clinical settings.

The initial feasibility studies combining the RosetteSep® CTC enrichment system with the ImageStream$^X$ demonstrated a combined yield of approximately 30%. Subsequent studies at Amnis have increased the yield of the enrichment process to approximately 90% and the fluidic system of the ImageStream$^X$ has been redesigned to achieve sample utilization efficiencies exceeding 95%. These two factors should result in a combined yield exceeding 90% and a CTC detection limit below 1 CTC/7 ml.