Endogenous light scattering as an optical signature of circulating tumor cell clusters

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Abstract: Circulating tumor cell clusters (CTCCs) are significantly more likely to form metastases than single tumor cells. We demonstrate the potential of backscatter-based flow cytometry (BSFC) to detect unique light scattering signatures of CTCCs in the blood of mice orthotopically implanted with breast cancer cells and treated with an anti-ADAM8 or a control antibody. Based on scattering detected at 405, 488, and 633 nm from blood samples flowing through microfluidic devices, we identified 14 CTCCs with large scattering peak widths and intensities, whose presence correlated strongly with metastasis. These initial studies demonstrate the potential to detect CTCCs via label-free BSFC.

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Metastases are the cause of 90% of annual cancer deaths in the U.S [1]. One of the main pathways that cells from the primary tumor use to establish distant metastases is through the blood circulation [2]. Thus, it is not surprising that circulating tumor cells (CTC) have been shown to correlate strongly with patient prognosis and metastasis formation in breast, prostate, and colon cancer patients [3, 4]. However, CTCs are rare compared to the over 5 billion red and 7.5 million white blood cells that are typically present in an mL of blood [5].
Currently the only FDA-approved CTC detection method for the clinic is the CellSearch® system (Veridex LLC, Warren, NJ, USA), which uses antibodies against Epithelial Cell Adhesion Molecule (EpCAM) for positive CTC detection and against CD45 for identification and depletion of leukocytes. Using CellSearch®, decreased progression free survival rates were found in metastatic breast cancer patients with >5 CTCs/7.5ml of whole blood [3]. However, further studies with this system led to detection of very low CTC counts in primary breast cancer patients and had poor correlation with prognosis, identifying its potential sensitivity limitations and inability to monitor or predict disease progression [6, 7]. Anti-EpCAM antibody based, microfluidic CTC capture devices have been developed to improve sensitivity, with the Herringbone Chip detecting a mean of 386 ± 238 CTCs per mL of blood in patients with metastatic prostate and lung cancer [8]. CTCs which have undergone epithelial-to-mesenchymal transition (EMT) may experience a downregulated expression of EpCAM on their surface [9] and this may limit their detection by this chip. Another microfluidic device, the CTC-iChip uses inertial flow to separate nucleated cells from whole blood and then employs CD45 and CD66b antibody coated magnetic beads to deplete white blood cells with a 97% yield of CTCs [10]. The high sensitivity of CTC detection using microfluidic devices has also enabled detection of even rarer CTC clusters (CTCCs), which appear to have stronger metastatic potential than single CTCs [8, 11]. While, the CTC-iChip eliminates the need for EpCAM, it still requires antibodies for negative depletion of WBCs, which may increase costs and lower the potential of translation in resource limited environments. Recently, microfluidic devices developed specifically for detecting CTCCs were able to identify clusters in 30%-40% of metastatic breast, prostate, and melanoma cancer patients without the use of antibodies [12].

CTCCs have also been detected in vivo using exogenous fluorescence based contrast. Methods that rely on endogenous sources of contrast for CTCC detection that can ultimately be translated to in-vivo studies haven’t been reported to our knowledge. Such methods have been developed for CTC detection and are based on endogenous fluorescence [13–18], coherent anti-stokes raman [19], and melanin photoacoustic signals [20].

In this study, we sought to identify the potential of backscattering flow cytometry (BSFC) to assess the number of CTCCs in response to a novel antibody-based treatment for triple negative breast cancers (TNBCs) in a mouse disease model. Specifically, the antibody targets the protein ADAM8, which is a transmembrane member of the A Disintegrin And Metalloprotease (ADAM) family of proteins that mediates cell adhesion, migration, and proteolysis of a variety of substrates, including cytokine receptors or their ligands, cell adhesion molecules and extracellular matrix components [21, 22]. ADAM8 is highly expressed in 34% of primary TNBC tumors, 50% of all breast cancer derived metastases, and is correlated with poor patient outcome [23]. In an orthotopic model, mice that were injected with green fluorescent protein-positive (GFP+) TNBC cells with an ADAM8 knockdown had smaller primary tumor growth and fewer and smaller metastases than mice injected with GFP+ TNBCs with functional ADAM8 [23]. Using a fluorescence based flow cytometer and a microfluidic platform, we found that green fluorescent cells (presumably GFP+ CTCs) were detected in significantly greater numbers in the blood drawn from mice implanted with functional ADAM8 TNBC cells compared to mice with ADAM8 knockdown TNBC cells at one, two, and three weeks following implantation [23]. Therefore, there was a strong correlation between the number of detected GFP+ cells and the growth of primary and secondary tumors. In this follow-up study, we initially aimed to assess the response of CTCs to antibody treatment relying on detection of their GFP fluorescence. In addition, we sought to identify CTC light scattering signatures that could be used to distinguish these cells from circulating white blood cells. The antibody treatment elicited the recruitment of cells in circulation that had strong green autofluorescence, limiting our ability to detect CTCs unambiguously. However, the combined fluorescence and light-scattering based flow cytometry measurements enabled the identification of a very rare population of events that we attribute to CTC clusters, whose number was strongly correlated with the presence of metastasis.
2. Methods

2.1 Mouse orthotopic model

Eleven non-obese, diabetic severe combined immunodeficiency (NOD/SCID) mice were implanted with $2.5 \times 10^6$ GFP+ clones of a known human triple negative metastatic breast cancer line, MDA-MB-231, in 50 $\mu$L of Matrigel in their mammary fat pads (MFP) [23]. Twelve days after inoculation, tumors became palpable, and treatment was initiated with 1.5 mg/kg of either an anti-ADAM8 antibody, Mab 1031 (R&D Systems) ($n = 6$, AA8), or an isotype-matched control antibody, IgG2B (R&D Systems) ($n = 5$, ISO). Three NOD/SCID mice were not implanted with tumors and used as background controls (non-cancerous, NC). The Mab 1031 antibody binds to the metalloprotease and disintegrin domains of the extracellular part of ADAM8, thus inhibiting the catalytic and signaling functions of these domains [21, 23]. Antibody was further administered on day 15 and then on day 17 when tumors reached a volume of ~0.15 $\text{cm}^3$ and tumor resection was performed. Antibody treatment was continued twice weekly for a five-week period, and then metastasis formation in the brain and lungs were examined using fluorescence microscopy [23].

2.2 Sample preparation

Blood from the ISO and AA8 treated mice was drawn using the submandibular blood collection method 50 days after inoculation (i.e., 37 days after tumor resection). As a background control, blood was also collected from three NC mice that were not injected with tumor cells. The blood volumes assessed were 198 ± 20 $\mu$L for NC ($n = 3$), 206 ± 84 $\mu$L for ISO ($n = 5$), and 251 ± 89 $\mu$L for AA8 ($n = 6$) mice. All blood samples were collected in heparin (100 USP units/sample) and processed the same day.

Blood samples were hemolyzed with EasySep Red Blood Cell Lysis Buffer (Stemcell Technologies, Vancouver Canada) and the remaining cells were re-suspended in RPMI-1640 without phenol red (Life Technologies, Woburn MA) at a concentration of 500,000 cells/ml determined using a haemocytometer. Samples were flowed in 30x30 $\mu$m² microfluidic channels made of polydimethylsiloxane (PDMS) with a glass bottom microscope slide, described previously [24]. Flexible tubing on one end of the devices was submerged in a cell solution constantly being stirred by a magnetic spin bar, while tubing on the other end was connected to a syringe pump pulling at 3 $\mu$L min$^{-1}$. Channels were pre-wetted by flowing phosphate buffered saline (PBS) and the cell suspension flow was allowed to stabilize prior to data acquisition. Samples were flowed for up to two hours or until there was no more cell solution.

2.3 Flow cytometer and data collection

The confocal backscatter and fluorescence flow cytometer has been described in greater detail by Greiner et al. [24]. Briefly, light from 405nm, 488nm, and 633nm lasers was directed into a single illumination path by mirrors and dichroic filters. The illumination beam was focused on a line by a cylindrical lens and re-imaged at the sample by an achromatic lens and an infinity corrected 40X microscope objective (NA 0.6; Olympus). Microfluidic devices were placed such that the illumination line traversed the channel through which samples flowed, as verified by a transillumination imaging setup. The backscattered and fluorescence light from flowing cells was collected by the same objective and imaged onto a 150 $\mu$m by 3000 $\mu$m slit aperture to minimize out of focus scattering. The angle of illumination was tilted between 0° and 18° allowing us to block the specularly reflected illumination beam and further reduce noise. Collected light was then separated by dichroic mirrors and directed on to one of four photomultiplier tubes (PMTs) with bandpass filters, to detect either scattered light at 405 ± 5 nm, 488 ± 5 nm, and 633 ± 5 nm, or fluorescence in the 500-590nm region. Data was sampled at 25KHz and digitally recorded as text files. All data processing was done in MATLAB (Natick, MA).
2.4 Data processing

Raw data was filtered by a 2nd order Butterworth bandpass filter from 30 Hz to 10 kHz to eliminate fluctuations in baseline intensities and high frequency detector noise. To enhance and simplify the peak identification algorithm, the signals from all scattering channels were used. Specifically, the signal from each channel was normalized by its standard deviation over the duration of data acquisition, and the intensities of the three channels were added to create the cumulative signal. The addition of signals from a cell event led to a larger signal to noise ratio, while spurious peaks tended to be averaged out. The cumulative signal was then analyzed using the MATLAB function findpeaks.m in the Signal Processing toolbox to identify peaks. Of the peaks identified, a cumulative signal threshold was applied so that the number of peaks with the highest signal was equal to the estimated number of cells that should have been counted. The latter number was approximated based on the cell concentration assessed by haemocytometer counts, the volumetric flow rate of 3μL/min, and the total acquisition time. To calibrate for day-to-day variations in instrument throughput, the 405, 488, and 633 nm scattering intensities were normalized for each cell peak by the mean intensity of the signal for that channel reflected by a spectralon reflectance standard [24].

A scattering peak was considered to be fluorescent if its peak fluorescence intensity had a value greater than the mean plus five times the standard deviation of the green fluorescence data trace intensity. The full width at half maximum (FWHM) of each cumulative signal peak was also measured. To assess the scattering properties of the different cell populations we detected, five derived parameters were compared, which included the ratio of peak intensities between scattering channels, i.e the 405/488, 405/633, 488/633 ratios, the cumulative channel intensity, and the spectral slopes, i.e. the slopes of line fits to the 405, 488, and 633 nm intensities for each peak.

2.5 Statistics

Mean ranks of the percentage of green fluorescent cells found within the different treatment groups were compared via a non-parametric Kruskal-Wallis test followed by a post-hoc Dunn’s multiple comparisons test appropriate for non-normal distributions [25], using the MATLAB 2014 Statistics and Machine Learning Toolbox. Kruskal-Wallis and post hoc Dunn’s tests were used to identify differences in the treatment group’s populations of fluorescent and non-fluorescent cells. Differences were tested in the mean ranks of the 405/488, 405/633, 488/633 ratios, cumulative intensities, green fluorescence peak FWHMs, and spectral slopes. To identify potential unique signatures of individual cells, the mahalanobis distance was used. To measure the mahalanobis distance MATLAB’s function mahal.m in the Statistics and Machine Learning Toolbox was used. The mahalanobis distance is an alternative classification method, well-suited for identification of outliers, and has been shown to be a robust classifier in multi-parameter flow cytometry [26]. It is analogous to the Euclidian distance between the center of a reference population and an individual event, with the distance between them being normalized by that population’s variance. The scattering ratios, cumulative intensity and FWHM of the corresponding NC fluorescent cells were used to calculate the mahalanobis distance. Rank-sum tests were performed to identify differences in the mean ranks of the ratios, cumulative intensity, green fluorescence intensity, spectral slopes, and FWHMs between the outlier peaks (as identified by the Mahalanobis distance) and all other fluorescent peaks.

3. Results

Representative flow cytometry data traces from which cell events were detected are shown in Fig. 1. Panel A shows a 2λ event, where the 405, and 488 channels have strong peak signals but the 633 channel has no detectable signal. In panel B, there is a 3λ event with strong 405, 488, and 633 signals but no fluorescent signal. A green fluorescent 4λ event, which has strong signal in all channels is shown in panel C. Finally, panel D shows an example of an extremely

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rare event, where all scattering channels exhibited saturated signals and large FWHMs. Rare events like these were primarily identified from mice which had formed metastases.

The average percentage of the cells that were green fluorescent for each treatment group is shown in Fig. 2. The small percentage of green fluorescent peaks detected in the NC group indicates that there is a group of normal circulating cells with strong green autofluorescence, since these mice weren’t implanted with any GFP+ cells.

Interestingly, the AA8 mice had a significantly higher mean rank for the percentage of green fluorescent cells compared to both ISO and NC mice (p<0.01), while there was no statistically significant differences in the percentage of green fluorescent cells of the NC and ISO mice. There was no difference in the mean peak fluorescence intensities between

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Fig. 1. Representative raw data time traces of a 2λ event (A), a 3λ event (B), a 4λ event (C), and a saturated and large FWHM 4λ event (D). The saturation output from the PMTs is 10V. For each panel, the top trace contains the scattering from the 405nm (black), 488nm (blue), and 633nm (red) scattering channels, the second and bottom traces contain the green fluorescence (green) and cumulative intensity (magenta) traces respectively.

Fig. 2. NC mice were found to have a small green autofluorescent population. Blood from the AA8 mice had a higher proportion of green fluorescent cells compared to both the ISO and NC groups (*p<0.01). ISO and NC mice did not have statistically significant differences in the proportion of detected green fluorescent cells.
treatment groups (p>0.05; data not shown). These results suggest that the majority of the green fluorescent cells detected in the blood of these mice represents one or more subpopulations of white blood cells that in the case of the AA8 group may have been activated in response to the antibody treatment.

The mean ranks of the peak intensity ratios for each treatment group’s fluorescent cells were not significantly different from each other or their corresponding non-fluorescent cells (p>0.05), as shown in Fig. 3 panels a-c. Medians of populations were marked by a circle, the 2nd and 3rd quartiles were marked by the ends of the boxes, and whiskers extended to the 1st and 4th quartiles. Outliers were plotted as separate points. For all treatment groups, green fluorescent cells had larger mean ranks for cumulative scattering intensities compared to non-fluorescent cells, as shown in Fig. 3 panel D (p<0.01). Although there were no significant differences in their mean-ranks, the fluorescent peaks of the ISO and AA8 groups had outliers with large FWHMs (Fig. 3(E)). There was no significant difference in the mean spectral slopes of the peaks from the different treatment groups (p>0.05), as shown in Fig. 3(F).

To further examine the potential presence of a population of circulating cells with distinct light scattering signatures, the mahalanobis distance of each cell from the centroid of the NC population was assessed. The distributions of mahalanobis distances of the fluorescent cells detected within each mouse are shown in Fig. 4 as box and whisker plots. A threshold was drawn to highlight the presence of fluorescent events which were extremely different in their scattering profiles from NC cells, thereby using the NC autofluorescent cells as a reference population. This revealed a total of fourteen extreme outliers. All extreme outliers were from mice with metastases, while 4/6 mice with metastases had extreme outliers revealing a strong correlation. Specifically, in the ISO control group, metastases to the brain and lungs were seen in 5/5 and 3/5 mice, respectively (two mice were not evaluated for lung metastases due to high autofluorescence). Out of six mice in the anti-ADAM8-treated antibody group, only one mouse showed a small metastasis in the brain. Interestingly, this mouse had two outlier events.

The 14 extreme outliers, plotted in red, have larger 405/488, 405/633, 488/633, and FWHM, compared to fluorescent non-outliers as shown in Fig. 5 and Table 1 (p<0.01). The size of the open circles in Fig. 5 corresponds to FWHM, with a larger circle having a larger value. The extreme outliers also had larger green fluorescence intensities and more negative
slopes compared to fluorescent non-outliers even though the latter difference was not statistically significant (p = 0.07). Since CTCs should be expressing GFP, a strong fluorophore, the finding that our extreme outliers are highly fluorescent based on their strong cumulative intensity suggests they may indeed consist of GFP + cancer cells. The finding that these extreme outliers have large FWHMs strongly indicates that they are clusters of cells.

Fig. 4. The Mahalanobis distance calculated for each fluorescent event detected based on the values of the peak intensity ratios, cumulative intensity and FWHM for NC (light grey), isotype (grey), and anti-ADAM8 treated mice (black).

Fig. 5. A 4 dimensional plot of the derived parameters reveals that fluorescent outliers (red) occupy a region with high 405/633, 488/633, 405/488, and increased FWHM scattering. Each axis is in log scale. The FWHM intensity is linearly signified by marker size. The 14 extreme outliers are plotted in red.

Table 1. Characteristics of Fluorescent Outliers and Fluorescent Non-outliers

|        | 405/488 | 405/633 | 488/633 | Cumulative | FWHM(ms) | Green Flr. | Slopes     |
|--------|---------|---------|---------|------------|----------|------------|------------|
| **Outliers** (14) | 2.2 ± 1.1* | 24.3 ± 7.4** | 13.7 ± 6.7** | 25.0 ± 12.9** | 3.4 ± 4.1** | 7.6 ± 8.4* | −2.80 ± 2.04E-3 |
| **Non-outliers** | 1.4 ± 0.9 | 4.4 ± 96.12 | 3.5 ± 43.5 | 3.5 ± 43.5 | 0.37 ± 0.83 | 2.8 ± 6.0 | −0.24 ± 0.58E-3 |

*,** indicate a significant difference at p<0.05 and p<0.01, respectively, between fluorescent outliers and fluorescent non-outliers by a rank-sum test.

4. Discussion

In this study, we demonstrate for the first time the potential of label-free detection of CTCCs based on the intensity and width characteristics of the backscattering signal detected from blood cells flowing through microfluidic devices. Specifically, we find CTCCs identified in the blood of 4 out of 6 mice bearing metastatic breast cancer tumors had significantly larger...
peak widths, cumulative intensities, and 405/488, 405/633, and 488/633 intensity ratios. As the signal we detect is collected through a confocal backscattering configuration (BSFC), there is great promise for translating this approach to label-free in vivo measurements. BSFC also offers a very attractive platform for the sensitive identification and isolation of rare CTCCs, potentially in combination with more sophisticated microfluidic platforms. Isolation and characterization of CTCCs is of great interest, as these cells may consist of cancer subpopulations that have unique properties and can be used as novel targets in preventing deadly metastases [11].

Along with the unique BSFC signatures for CTCCs, we detected in this study a significant increase in the number of circulating autofluorescent cells in the group of mice treated with the anti-ADAM8 antibody. While the presence of these cells hindered our ability to assess the effect of this treatment on the prevalence of CTCs, it suggests that endogenous fluorescence based in vitro and in vivo flow cytometry may provide a label-free, ultimately non-invasive method for assessing the efficacy of cancer immunotherapies in mobilizing an immune response. Thus, in future studies it will be interesting to explore this use of fluorescence based FC to perform experiments designed to characterize the identity of these mobilized autofluorescent cells.

The number of mobilized red autofluorescent cells, relative to the number of green, likely GFP + circulating breast cancer cells has also been found to correlate with metastatic potential in a previous IVFC study monitoring in vivo the blood of mice implanted with SUM1315 breast cancer cells [16]. Such autofluorescent cells consisted at least in part of CD31+ precursor endothelial cells (39%) and sca-1+ cells (18%), which may be immature leukocytes, natural killer (NK) cells, macrophages, or granulocytes. Since the mice we used in this study lack NK cells, and mature T and B lymphocytes, the autofluorescent cells we detected may be immature leukocytes. Monocytes, neutrophils, and eosinophils in human peripheral blood have been reported to be autofluorescent [16, 27–29]. The source of this autofluorescence is likely due to riboflavin, and flavins like FAD [30, 31]. Thus, the increase in fluorescent cells due to anti-ADAM8 antibody therapy could also be due to a population of monocytes, neutrophils, or eosinophils.

In summary, our findings demonstrate the potential of label-free based flow cytometry methods to detect subpopulations of circulating cells and cell clusters that may serve as useful tools for developing new cancer therapies and monitoring their efficacy. Exploitation of the endogenous fluorescence and light scattering signals can be easily combined with in-vitro based platforms that rely on size-based and/or antibody-based approaches to detect specific cell populations of interest with enhanced sensitivity and specificity. Translation to in vivo studies is also possible and offers unique opportunities for monitoring CTCCs or other mobilized cell populations in a painless and artifact free manner.

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