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Abstract. The identification of breast cancer patients who will ultimately progress to metastatic disease is of significant clinical importance. The quantification and assessment of circulating tumor cells (CTCs) has been proposed as one strategy to monitor treatment effectiveness and disease prognosis. However, CTCs have been an elusive population of cells to study because of their small number and difficulties associated with isolation protocols. *In vivo* flow cytometry (IVFC) can overcome these limitations and provide insights in the role these cells play during primary and metastatic tumor growth. In this study, we used two-color IVFC to examine, for up to ten weeks following orthotopic implantation, changes in the number of circulating human breast cells expressing GFP and a population of circulating hematopoietic cells with strong autofluorescence. We found that the number of detected CTCs in combination with the number of red autofluorescent cells (650 to 690 nm) during the first seven days following implantation was predictive in development of tumor formation and metastasis eight weeks later. These results suggest that the combined detection of these two cell populations could offer a novel approach in the monitoring and prognosis of breast cancer progression, which in turn could aid significantly in their effective treatment. © 2011 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.3560624]

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In the United States, breast cancer is the second leading cause of cancer death among women, with approximately 180,000 new cases and 40,000 deaths occurring yearly.¹ Immunomagnetic bead isolation,² standard flow cytometry,³ and CTC-chip technology⁴ have been used to quantify circulating tumor cells

(CTCs) in patients with metastatic breast cancer. These measurements have demonstrated that the presence of more than 5 breast cancer cells per 7.5 mL of isolated blood correlates highly with poor patient prognosis.² While these studies have shown promise, they require isolating peripheral blood from cancer patients and are labor intensive, involving processing steps that can eliminate already rare cancer cells.

An optical technique that could address these limitations is *in vivo* flow cytometry (IVFC). Fluorescence-based IVFC can detect quantitatively fluorescent cells that flow in the microvasculature of small animals. White and red blood cells,⁵ stem cells,⁶ leukemic cells,^{7,8} and breast,^{6,9} ovarian,⁸ and prostate cancer cells¹⁰ have all been detected *in vivo* using IVFC. As this method enables noninvasive detection of cells within their natural environment, and allows for multiple measurements on the same subject without disturbing natural dynamic cell interactions, it is an ideal approach for studying rare circulating cells.

In this study, we utilized two-color IVFC⁶ to excite circulating cells at 488 nm (for GFP excitation) and at 633 nm (for autofluorescence excitation). We acquired measurements prior to, and following implantation of GFP-labeled fibroblasts (RMF/EG-control group) or human breast cancer cells (SUM1315 or DU4475) into the mammary glands of nonobese diabetic severe combined immuno-deficient (NOD/SCID) mice. We found that the combined use of the number of CTCs and autofluorescent cells can be correlated with the development of primary and metastatic tumor growth. Initial antibody experiments suggest that these autofluorescent cells are a population of circulating immature leukocytes (CD31 + and/or Sca-1 +).

Specifically, 8 to 12 week old female NOD/SCID mice were used for this experiment. Prior to surgical implantation, the cells (RMF/EG immortalized human breast fibroblasts, used as controls, and SUM1315 and DU4475 human breast cancer cells) were suspended in a 4:1 volume mixture of culture medium and Matrigel (BD Bioscience). One million cells were injected in the 4th inguinal mammary gland of each mouse. Five mice were injected with SUM1315 cells, six mice were injected with DU4475 cells, and four control mice were injected with RMF/EG cells. One mouse was not injected with any cells. Measurements were taken twice a week on each mouse for eight to eleven weeks post implantation or until tumors reached 2 cm³ in volume. All animal procedures were approved by the institutional animal care and use committee of Tufts University.

The setup of the two-color IVFC has been described previously.⁶ Approximately 30 μ m arteries were selected for data acquisition. Light from a 488 nm diode pumped solid state laser and a 633 nm HeNe laser were shaped into a slit and imaged across the artery. Fluorescence emitted at 510 to 590 nm and at 650 to 690 nm was detected confocally. For most mice, baseline measurements were obtained one to three times prior to surgery, within the hour, and every third or fourth day following surgery. The average number of detected fluorescent cells per microliter of blood was recorded. The blood volume was assessed based on the diameter of the blood vessel and the width of the optical slit on the vessel, as visualized by the transillumination setup, and the full width at half maximum of the fluorescence peaks.

Representative peaks detected by the green fluorescence channel (510 to 590 nm) are shown in Fig. 1(a). Red

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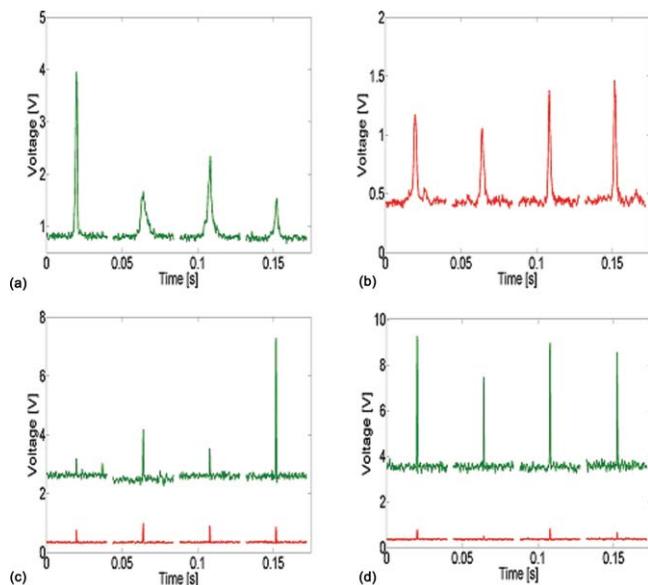


Fig. 1 IVFC signals recorded from (a) GFP-expressing human breast cancer cells, and (b) red autofluorescent cells flowing through the artery of a mouse ear. Correlated signals recorded from two photodetectors when red autofluorescent cells (red) detected within mouse blood are tagged with either (c) anti-cd31 FITC antibody (green) or (d) anti-sca1 FITC antibody (green).

autofluorescent cells (650 to 690 nm) are also detected within arteries of each mouse [Fig. 1(b)]. To determine the identity of the red autofluorescent cells, we performed a single set of *in vitro* antibody labeling experiments using the pooled blood of six of the tumor bearing mice of our study. Specifically, 220 μL of diluted mouse blood (1:3 volume mixture of whole blood to DMEM) was mixed with 5 μL of 0.5 mg/mL FITC anti-mouse cd31, or FITC anti-mouse sca-1 antibody (220 μL of blood without any antibody served as a control). The mixtures were incubated at 4°C in the dark for 30 min and flowed through 70 μm single channel polydimethylsiloxane (PDMS) microfluidic devices. Measurements were taken on the samples with the IVFC instrument used for the animal studies. Examples of red autofluorescent peaks colabeled with antibodies against CD31-FITC or Sca-1 FITC are shown in Figs. 1(c) and 1(d), respectively. In this experiment, approximately 39% of the CD31-positive and 18%

of the Sca-1-positive peaks were correlated with the red autofluorescent cells. These antibodies target precursor endothelial cells and immature leukocytes as well as other hematopoietic cell populations such as natural killer (NK) cells, macrophages, and granulocytes.^{11,12} While further controls to account for potential nonspecific binding and repeat experiments are needed to confirm the identity of these cells, our initial measurements suggest that they are likely a heterogeneous subpopulation of immature leukocytes.

To assess the presence of metastasis, tissue from the lung, brain, and tumor were harvested from each mouse upon sacrifice. Any tissue from the lung or brain that emitted green fluorescence as seen under a fluorescence microscope (4 \times magnification) indicated metastasis [Fig. 2(a)]. Primary tumor sections were also assessed histologically. Tumors that showed cancer cells forming a tight border around the stroma indicated low to no potential that metastasis had occurred [Fig. 2(c)]. In contrast, tumors that showed cancer cells invading through the border and into the stroma, indicated high potential that metastasis had occurred [Fig. 2(b)].

Based on this assessment, we show in Fig. 3 the mean number of green and red autofluorescent cells from five groups of mice: a. control mice implanted with fibroblasts ($n=4$) or no cells ($n=1$), b. mice implanted with DU4475 cells that did not develop a primary or a metastatic tumor ($n=4$), c. mice implanted with DU4475 cells that did develop a primary and a metastatic tumor ($n=2$), d. mice implanted with SUM1315 cells that developed a primary tumor but no metastasis ($n=3$), and e. mice implanted with SUM1315 cells that developed both a primary and a metastatic tumor ($n=1$). The average number of green fluorescent cells detected on each measurement day for each group of mice is shown in Fig. 3(a). During the first few days post surgery, the number of such cells is significantly higher for mice that eventually develop tumors compared to those that do not. This suggests that successful tumor cell implantation may be predicted with IVFC within the first seven to ten days and in most cases several weeks before a palpable tumor is observed.

The average ratio of green to red fluorescent cells for each group of mice over the same period is shown in Fig. 3(b). Even though the number of mice included in this study is small, this ratio is strikingly higher during the first two weeks following implantation for the mice that develop metastatic tumors than the mice that do not. Therefore, this ratio may be a promising

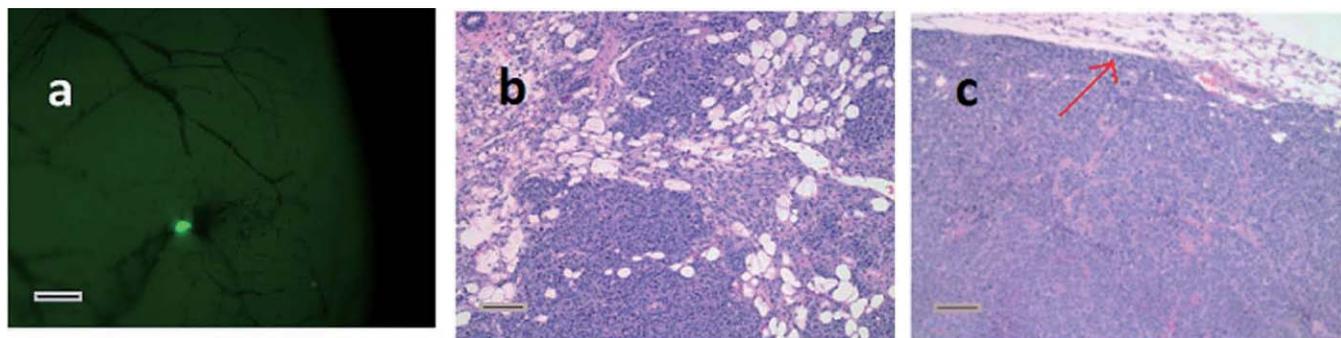


Fig. 2 (a) Fluorescence image from whole mount of brain tissue showing metastasis of GFP-expressing SUM1315 cells. Scalebar = 583 μm . (b) H&E stained section of a SUM1315 primary tumor that shows invasion of cancer cells (blue) into the adipose tissue (white), which suggests metastatic potential. Scalebar = 100 μm . (c) H&E stained section of a SUM1315 primary tumor that does not show invasion of cancer cells, which suggests no metastatic potential. Red arrow points to boundary between adipose tissue and cancer cells that have not invaded. Scalebar = 100 μm .

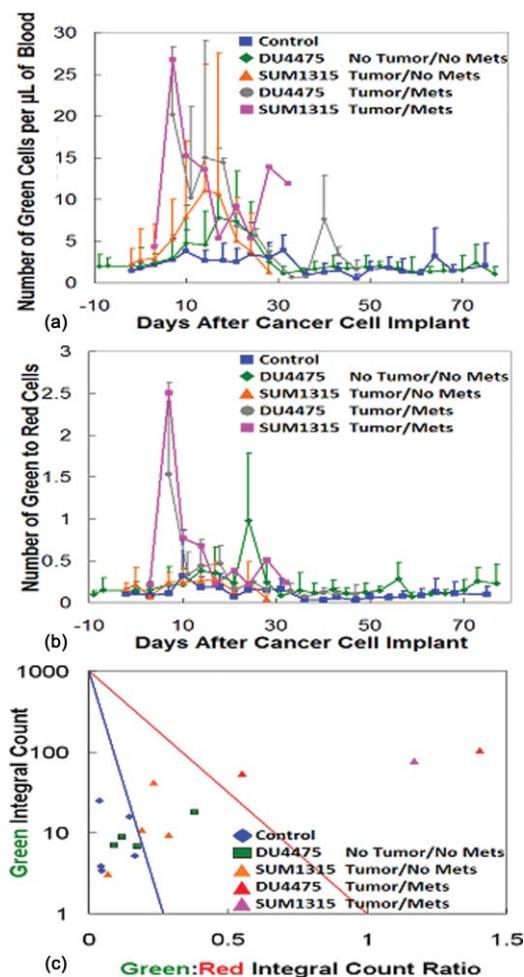


Fig. 3 Changes in (a) the average number of circulating green fluorescent cells, and (b) the average ratio of circulating green to red fluorescent cells detected between each group of mice as a function of time before and after cancer cell implantation. Standard deviations are shown for each group. (c) The integrated number of green fluorescent peaks to the ratio of green to red fluorescent peaks detected during the first seven days as an indicator of tumor progression and metastasis. A threshold line separates mice that developed tumors from mice that did not (blue) and mice that developed metastasis from mice that did not (red).

indicator of the metastatic potential of a tumor. In addition, at least for this type of tumors, the number of circulating cells at the very early stages of primary tumor development are highly predictive of the formation of micro-metastasis detected several weeks later.

Specifically, we find that the integrated number of green fluorescent peaks to the ratio of green to red fluorescent peaks within the first seven days following cell implantation offers the most accurate discrimination of the mice in groups with: a. no tumor, b. primary tumor development and no metastasis, and c. primary and metastatic tumor growth. Using the simple lines of Fig. 3(c) drawn by visual inspection of the data, one could separate 3/3 mice with metastasis, 6/7 mice with tumors and 8/9 mice with no tumor.

In a previous study that used multiphoton flow cytometry, rare cancer cells were also detected in the microvasculature of

mice.⁸ The results indicated an exponential increase in lung cancer cell count within four weeks after implantation using folate fluorescent dyes to tag the cells. Such an exponential growth in the number of circulating cells is not observed in our study, suggesting that the kinetics of CTCs are likely to be very specific to the type of cancer cell involved. In fact, in our study the combined use of CTCs and immature leukocytes appears a more diagnostically useful approach than the number of CTCs alone.

In summary, our study demonstrates that multicolor IVFC may offer highly novel and useful insights in the role of circulating cancer and immune system cells in primary and metastatic tumor development. This initial study includes a small number of animals and it will be important to confirm these findings in a larger scale study to explore the potential of this approach as a predictor of cancer development and/or response to treatment. In addition, the precise identification of the autofluorescent cells as well as the development of methods to exploit their numbers in circulation may provide improved therapeutic approaches.

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