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Monitoring circulating cancer cells by multichannel in vivo flow cytometry

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***Abstract*—We report a new approach for potential monitoring of tumor burden in experimental animals using multichannel *in vivo* flow cytometry, a novel optical technique that enables the real-time, continuous detection and quantification of fluorescently labeled cells in the circulation without the need for blood extraction. The ability to non-invasively track circulating cells in real time and in their native environment, opens up enormous possibilities for new investigations into the mechanisms that govern the complex trafficking and tissue interactions of these cells in a wide range of clinical and biological fields such as cancer, stem cell biology and immunology. We have developed the *in vivo* flow cytometer in order to track circulating cancer cells in a mouse model and provide a new, non-invasive method for the monitoring of cancer disease progression as well as the response to therapeutic intervention.**

Keywords—circulating cancer cells, in vivo flow cytometry

I. INTRODUCTION

In vivo measurement of tumor burden, both in cancer research models and in patients, is an important parameter for the accurate assessment of disease progression and the response to therapeutic intervention [1]. Several in vivo imaging modalities have been utilized in the assessment of tumor burden, including functional magnetic resonance imaging, computer tomography and positron emission tomography [2, 3], fluorescence imaging [4, 5], intravital microscopy [6] and bioluminescence imaging [7, 8]. More recently, the detection and quantification of circulating cancer cells has been explored as a method to evaluate tumor burden in the context of assessing disease stage, prognosis as well as monitoring disease progression following therapeutic intervention in cancer patients [9, 10]. Clinically, various ex vivo assays have been developed to detect cancer cells shed in circulation by primary tumors, including breast cancer, prostate cancer and small-cell lung cancer [11, 12, 13]. *In vivo* flow cytometry has been developed as a method for real-time detection of circulating cancer cells injected into the circulation of experimental animals [14,15,16]. The method does not require extraction of blood samples and is therefore well suited for long-term monitoring of circulating tumor cells. In this report, we report on the development of a multichannel *in vivo* flow cytometer to detect and quantify circulating cancer cells as a means of assessing the tumor burden in animal models.

II. MATERIALS AND METHODS

A. Development of a multichannel in vivo flow cytometer

Multichannel *in vivo* flow cytometry combines the principles of confocal detection and flow cytometry in order to enable the real-time detection of fluorescently labeled cells circulating in a live animal. The system can be applied for the dynamic and simultaneous monitoring of multiple populations of circulating cells, which can be targeted and labeled with multiple fluorescent markers and probes. To accomplish this, light from up to three separate excitation lasers was focused by a cylindrical lens and then imaged across a blood vessel to form an excitation slit. Each laser beam slit was about 70 μm x 5 μm in size, with a depth of focus of about 50 μm (in the axial direction). Figure 1 illustrates the concept of the multichannel in vivo flow cytometer (shown here, for demonstration purposes, with two rather than three detection channels). When the cells, labeled with an exogenous fluorescent marker were flown through the excitation slit, the fluorescence signal emitted was confocally detected by a photomultiplier tube in each of the detection channels of the system. The collected signal was then sent to an analog-to-digital converter to be digitized and stored on a computer for analysis. PMT signal traces were then analyzed by Matlab software to identify cell peaks and extract quantitative information on the number of cells passing through the excitation slits.

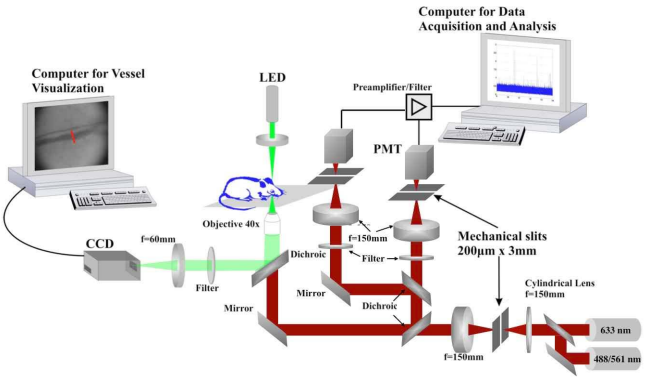

Figure 1. Schematic of the multichannel in vivo flow cytometer

The excitation lasers chosen were a 633 nm Helium-Neon laser (JDS Uniphase, Milpitas, CA, USA), due to its good penetrating capability through tissue and blood, as well as 561 nm (Cobolt AB, Solna, Sweden) and 488 nm (Coherent Inc, Santa Clara, CA, USA) diode-pumped, solid-state lasers, due to their strong excitation capability of commonly used fluorochromes such as green fluorescent protein (GFP), fluorescein, and the yellow/red fluorescent protein variants (YFP/RFP). Arteries (with average diameter of 40-50 μm), rather than veins, were chosen for experiments because of faster blood flow and absence of cell-endothelium interactions.

B. Application of the system for in vivo cell detection

The fluorescence markers selected in order to label cells for *in vivo* detection were the lipophilic carbocyanine dyes Vybrant DiO, DiI and DiD (Life Technologies, Grand Island, NY, USA). 10^6 cells/ml from the MDA-MB-231 breast adenocarcinoma cell line were incubated separately with the Vybrant fluorescent probes (each at a concentration of 5 μg/ml) for 20-30 minutes in order to achieve uniform staining of the cell membrane. The fluorescently labeled cells were first flown through a microfluidic channel (diameter 100 μm) in order to validate the fluorescent detection capabilities of the system. The 633 nm HeNe laser (JDS Uniphase, USA) was employed to excite the Vybrant DiD fluorescence of the flowing cancer cells while the 488 nm and 561 nm lasers were used for the excitation of the Vybrant DiO and DiI probes respectively. Signal was confocally detected in each detection channel by a photomultiplier tube (Hamamatsu, Hamamatsu, Japan) after passing through a bandpass filter, and was then digitized for analysis on a PC equipped with Matlab software. Characteristic fluorescence peaks in the PMT signal due to the detection of individual flowing DiD-labeled cells are shown in Figure 2. Fluorescently labeled MDA-MB-231 cells were then separately injected through the tail vein of anesthetized male, 10-12 week old, CD-1 mice (Cyprus Institute of Neurology and Genetics) at approximately $2x10^6$ cells per mouse, in order to demonstrate the *in vivo* capabilities of the system. The anesthetized animals were placed on an imaging platform within 5 minutes after injection of the cells and an appropriate arteriole in the ear was chosen from which to obtain circulating cell count measurements. A characteristic PMT signal of *in vivo* detected circulating cancer cells is shown in Figure 3. Due to the rapid clearance of these cells from circulation, only a few cells were observed during the first few minutes following tail vein injection, however circulating cells were clearly observed in all the mice that had cells successfully introduced in the vasculature.

III. RESULTS AND DISCUSSION

We have developed the multichannel *in vivo* flow cytometer for the dynamic monitoring of cells in circulation. The system was designed and built with the ability to simultaneously assess the circulation kinetics of several distinct cell populations in a single animal. This will allow for a more efficient *in vivo* investigation of complex biological processes by enabling the simultaneous monitoring of the multiple cell populations that might be participating and interacting in such processes. Here we have reported on the development of the multichannel *in vivo* flow cytometer and have demonstrated its capabilities in detecting and quantifying fluorescently labeled breast adenocarcinoma cells directly injected in the circulation of experimental animals.

To better approximate the tumor environment *in vivo*, a mouse tumor model will be developed in which breast cancer cells will be adoptively transferred in mice and allowed to migrate to tumor growth areas. Instead of labeling them with exogenous fluorescent dyes, these cells will be engineered to express a fluorescent protein. The cells will then be injected in immune-compromised mice and allowed to grow tumors. Once the tumors are established, the animals will be monitored long term using the multichannel *in vivo* flow cytometer in order to quantify the fluorescent tumor-shed cells in circulation. Tumor burden will also be assessed via already established methods (such as histology) and the results will be

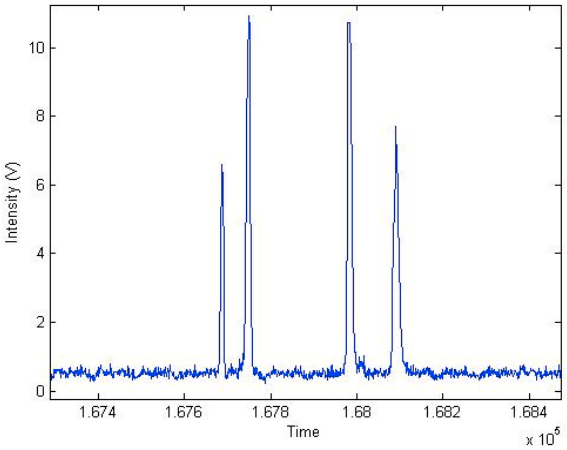

Figure 2. Breast adenocarcinoma cells labeled with the Vybrant DiD fluorescent probe and detected in vitro (microfluidic channel)

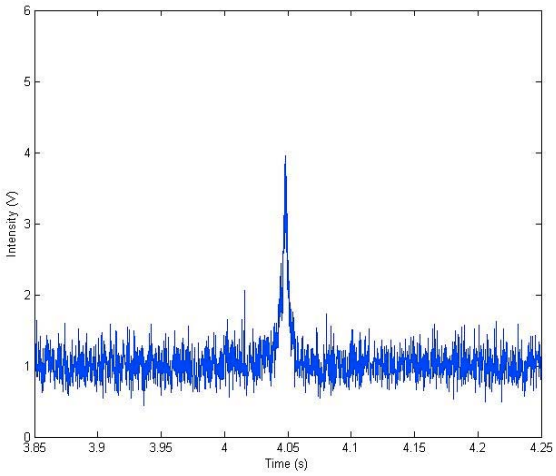

Figure 3. Breast adenocarcinoma cells labeled with the Vybrant DiD fluorescent probe and detected in vivo (CD-1 mouse)

compared to data on circulating cancer cells in order to validate the method for the *in vivo* assessment of tumor burden in animals. By quantifying circulating tumor cells, in cancer disease models that include a circulating cell component, the *in vivo* flow cytometer can be used to non- invasively track tumor burden and thus assess important cancer treatment parameters such as the tumor growth and the response to therapeutic intervention.

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REFERENCES

[1] G. P. Schmidt, H. Kramer, M. F. Reiser, and C. Glaser, "Whole-body magnetic resonance imaging and positron emission tomography-computed tomography in oncology," Top. Magn. Reson. Imaging, vol. 18, no.3, pp. 193-202, Jun. 2007.

[2] T. Beyer, et al, "A combined PET/CT scanner for clinical oncology," J. Nucl. Med., vol. 41, no. 8, pp. 1369-1379, Aug. 2000.

[3] H. U. Kauczor, C. Zechmann, B. Stieltjes, and M. A. Weber, "Functional magnetic resonance imaging for defining the biological target volume," Cancer Imaging, vol. 6, no. 1, pp. 51-55, Jun. 2006.

[4] C. S. Mitsiades, et al, "Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD in vivo model: biologic and clinical implications," Cancer Res., vol. 63, no. 20, pp. 6689-6696, Oct. 2003.

[5] H. Yamamoto, et al, "Quantitative assessment of small intraosseous prostate cancer burden in SCID mice using fluorescence imaging," Prostate, vol. 67, no. 1, pp. 107-114, Jan. 2007.

[6] J. Condeelis and J. E. Segall, "Intravital imaging of cell movement in tumours," Nat. Rev. Cancer, vol. 3, no. 12, pp. 921-930, Dec. 2003.

[7] C. H. Contag, D. Jenkins, P. R. Contag, and R. S. Negrin, "Use of reporter genes for optical measurements of neoplastic disease in vivo," Neoplasia, vol. 2, no. 1-2, pp. 41-52, Jan. 2000.

[8] C. M. Deroose, et al, "Multimodality imaging of tumor xenografts and metastases in mice with combined small-animal PET, small-animal CT, and bioluminescence imaging," J. Nucl. Med., vol. 48, no. 2, pp. 295-303, Feb. 2007.

[9] M. Cristofanilli, "Circulating tumor cells, disease progression, and survival in metastatic breast cancer," Semin. Oncol., vol. 33, no. 3, pp. S9-14, Jun. 2006.

[10] J. B. Smerage and D. F. Hayes, "The measurement and therapeutic implications of circulating tumour cells in breast cancer," Br. J. Cancer, vol. 94, no. 1, pp. 8-12, Jan. 2006.

[11] Y. P. Sher, et al, "Prognosis of non-small cell lung cancer patients by detecting circulating cancer cells in the peripheral blood with multiple marker genes," Clin. Cancer Res., vol. 11, no. 1, pp. 173-179, Jan. 2005.

[12] Z. P. Wang, et al, "Identification and characterization of circulating prostate carcinoma cells," Cancer, vol. 88, no. 12, pp. 2787-2795, Jun. 2000.

[13] K. Pachmann, et al, "Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse," J. Clin. Oncol., vol. 26, no. 8, pp. 1208-1215, Mar. 2008.

[14] X. Wei, et al, "Real-time detection of circulating apoptotic cells by in vivo flow cytometry," Mol. Imaging, vol. 4, no.4, pp. 415-416, Oct. 2005.

[15] S. Boutrus, et al, "Portable two-color in vivo flow cytometer for real-time detection of fluorescently-labeled circulating cells," J. Biomed. Opt., vol. 12, no. 2, pp. 020507, Mar. 2007.

[16] E. R. Tkaczyk, et al, "In vivo monitoring of multiple circulating cell populations using two-photon flow cytometry," Opt. Commun., vol. 281, no. 4, pp. 888-894, Feb. 2008.