Studying circulation times of liver cancer cells by in vivo flow cytometry

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Abstract. Hepatocellular carcinoma (HCC) may metastasize to lung, bones, kidney, and many other organs. The survival rate is almost zero for metastatic HCC patients. Molecular mechanisms of HCC metastasis need to be understood better and new therapies must be developed. A recently developed “in vivo flow cytometer” combined with real-time confocal fluorescence imaging are used to assess spreading and the circulation kinetics of liver tumor cells. The in vivo flow cytometer has the capability to detect and quantify continuously the number and flow characteristics of fluorescently labeled cells in vivo in real time without extracting blood sample. We have measured the depletion kinetics of two related human HCC cell lines, high-metastatic HCCLM3 cells and low-metastatic HepG2 cells, which were from the same origin and obtained by repetitive screenings in mice. >60% HCCLM3 cells are depleted within the first hour. Interestingly, the low-metastatic HepG2 cells possess noticeably slower depletion kinetics. In comparison, <40% HepG2 cells are depleted within the first hour. The differences in depletion kinetics might provide insights into early metastasis processes.

1. Introduction
Liver cancer is one of the most common malignancies in the world, with approximately 1,000,000 cases reported every year. Hepatocellular carcinoma may metastasize to lung, bones, kidney, and many other organs [1-5]. Surgical resection, liver transplantation, chemotherapy and radiation therapy are the foundation of current HCC therapies. However the outcomes are poor: the survival rate is almost zero for metastatic HCC patients.

Metastasis is a very complicated process that still has yet to be completely understood. In metastasis, the cancer cells that travel through the body are capable of establishing new tumor sites in locations remote from the site of the original disease. To metastasize, a cancer cell must break away
from its tumor, invade either the circulatory or lymph system, which will carry it to a new location, and establish itself in the new site [6]. Molecular mechanisms of HCC metastasis need to be understood better and new therapies must be developed to selectively target to unique characteristics of HCC cell growth and metastasis.

Conventionally, researchers extract samples of tumor tissue or blood from patients or animal models to study the mechanisms of HCC metastasis with biochemical analysis, along with extremely careful protocols to reduce the changes induced by sample extraction [7]. In order to reveal the exact mechanisms in certain circumstances, there is a great demand to monitor these molecular events in vivo, where modern optical techniques became more and more powerful.

Existing optical techniques including confocal microscopy, multi-photon microscopy [8], optical coherence tomography (OCT) [9], and so on are mainly imaging methods that give insight into real-time biological reactions or phenomenon. However, quantitative evaluations of such biological processes are crucial to discover mechanisms, for example cancer metastasis.

Flow cytometer was developed to analyze quantitative characteristics about cells in vitro, by flowing single cells through fine tube [10]. Cells are suspended in a stream of fluid and one by one pass the focus of a laser light beam or several laser light beams. On the excitation, fluorescent signals and other scattering characteristics will be immediately collected by the various detectors and later processed to analyze the numbers of different cells groups. Inspired from this technique and principles of confocal microscopy, scientists invented “in vivo flow cytometer” and applied it to various biological studies [11-13]. The blood flow within micro-vessel in the ear (or other accessible part) of small animals is used similarly as the stream of fluid in flow cytometer. Labelled cells passing through linearly focused laser beam are excited by certain laser and then emit specific fluorescence to be detected. Up to date, there are a number types of “in vivo flow cytometers” developed, i.e. two-photon [14-16], multi-color [11, 12, 17], photoacoustic [17-20] in vivo flow cytometer, for the biological studies varying from cancer cells to immune cells for quantitatively analysing kinetics of cells in either the blood or lymphatic circulation system.

In order to study characteristics of circulating HCC cells, here we apply the “in vivo flow cytometry” combined with optical imaging to study if there is any relationship between kinetics of circulating tumor cells (CTCs) and its metastatic potential.

2. Materials and methods

2.1. In vivo flow cytometry
The in vivo flow cytometer has the capability to detect and quantify continuously the number and flow characteristics of fluorescently labeled cells in vivo [11-13, 21-24]. The in vivo flow cytometer allows researchers to acquire cytometric information from the circulation in live animals without extracting blood sample (Figure 1). Previous researchers demonstrate that using this technique, the number of fluorescently-labeled circulating cells can be quantified in a real-time and reproducible manner in live animal. We set up our in vivo flow cytometer to detect fluorescence signal from a given circulating tumor cell (CTC) population in a confocal geometry based on previous groups’ experiences [11, 13].

Briefly, fluorescence signal from a given circulating cell population is recorded as the cells pass through the slit of light. Confocal detection of the excited fluorescence enables continuous monitoring
of labeled cells in the upper layers of scattering tissue, such as the skin of a mouse ear. The size of the slit at the focal plane of the sample is approximately 5x72 µm. The depth of focus (i.e. the full width at half maximum of the light slit onto the sample in the axial direction) is approximately 50 µm, a value chosen to match the vessels of interest. The sample is positioned so that the long dimension of the slit traverses the width of the blood vessel; thus, fluorescence is excited as the labeled cells in circulation pass through the slit. Fluorescence is detected with a photomultiplier tube placed directly behind the mechanical slit, sampled at a rate of 5 kHz with a data acquisition card, and displayed and stored on a computer. The device has been used to characterize the in vivo kinetics of red and white blood cells circulating in the mouse ear vasculature, as well as in the circulating liver cancer cells.

Figure 1. Schematic of the two-color in vivo flow cytometer experimental setup. Laser light (488 nm or 635 nm) is focused into a slit by a cylindrical lens (CL) and imaged across the selected blood vessel with a microscope objective lens (40X, 0.6 NA). The fluorescence is collected by the same microscope objective, directed through the dichroic beam splitter BS3 (reflection 25%, transmission 75%, Edmund Optics), reflected by a mirror, a second splitter BS2 (transmission 90% for 488 nm and 635 nm; reflection 90% for 499-622 nm, and 652-755 nm, Semrock) and a third dichroic beam splitter BS4 (edge wavelength 516 nm, reflection band >90% for 490-510 nm, transmission band >90% for 520-700 nm, Semrock), and imaged onto a 200 µm x 3000 µm mechanical slit, which is confocal with the excitation slit. F1-3: bandpass filter (F1: 509-552 nm; F2: 500-520 nm; F3: 640-690 nm; Semrock). BS1: beam splitter, edge wavelength 505 nm, reflection band 505 nm, transmission band 446-500 nm, Semrock). AL1-3: achromatic lenses.

2.2. Animal experiments
In experiments, the BALB/c nude mice were anesthetized and injected intravenously with DiD-fluorescently-labeled HCCLM3 human liver tumor cells. The HCCLM3 is a hepatocellular carcinoma (HCC) cell line with high metastatic potential and comparatively easier to metastasize
to the lung in BALB/c nude mice than other cell lines [25]. Studying the depletion kinetics of HCCLM3 cells in the circulation could help understand the early metastatic process. After the injection, mice were then placed on a heated stage and an artery in the ear was chosen for obtaining measurements. Fluorescence signal was excited as the labeled circulating cells passed through a laser slit focused across the blood vessel (Figure 2). Detecting the excited fluorescence confocally enables researchers to monitor labeled cells in the animal circulation system continuously. Signal was recorded at a rapid rate (5 KHz) to ensure the measurement of fast-flowing cells (Figure 3). The number of fluorescent peaks, along with the height and full width at half maximum of each peak, was determined using algorithms developed in-house. The device has been used to characterize the in vivo kinetics of red and white blood cells circulating in the mouse ear vasculature as well as the circulating prostate cancer cells [11-13, 26].

**Figure 2.** The mouse is anesthetized and placed on a heated stage. An artery is chosen in the ear for the measurement. The fluorescence signal from DiD-labeled circulating cells is recorded when the cells pass through a slit of light focused across the artery. DiD is a fluorescent dye to label the membrane lipid (excitation peak: 640nm; emission peak: 660-670nm; Molecular Probes, USA).
Figure 3. A trace of labeled circulating HCCLM3 hepatocellular carcinoma cell measured by the in vivo flow cytometer, after intravenously introduced into a mouse. The peak within the trace indicates that a DiD-labeled HCCLM3 cell passes through the slit of light and thus gives a burst of fluorescence.

The sample isolation and preparation steps here are bypassed altogether in contrast to the measurement of a typical conventional (in-vitro) flow cytometer. Multiple measurements in the in-vivo-flow-cytometer are carried out noninvasively. We have measured the depletion kinetics of two related human HCC cell lines, high-metastatic HCCLM3 cells and low-metastatic HepG2 cells, which were from the same HCC patient and obtained by repetitive screenings in mice [25].

3. Results

The depletion kinetics of circulating HCCLM3 cells in BALB/c nude mice during the first 12 hours following injection of the fluorescently labeled cells illustrates the depletion process (Figure 4). >60% cells are depleted within the first hour. After the initial depletion, there is a re-appearance in the number of circulating cells, quickly followed by a second depletion. This phenomenon was also observed in previous study on prostate cancer cells [26]. By 12 hours, ~94% HCCLM3 cells are depleted from the circulation. Interestingly, the low-metastatic HepG2 cells possess noticeably slower depletion kinetics. In comparison, <40% HepG2 cells are depleted within the first hour. By 12 hours, ~55% HepG2 cells are depleted from the circulation. When present in circulation for extensively long time, cancer cell might undergo cell death due to lack of survival signal from cell adhesion and the harsh environment imparted by the sheer stress [27, 28]. Therefore, the differences in depletion kinetics might provide insights into early metastasis processes.
Figure 4. Depletion kinetics of circulating high-metastatic HCCLM3 cells and low-metastatic HepG2 cells in BALB/c nude mice. The normalized numbers of circulating cells per min are shown for 12 hours following injection of the DiD-fluorescently-labeled cells to illustrate the depletion process. >60% HCCLM3 cells are depleted within the first hour. By 12 hours, ~94% HCCLM3 cells are depleted from the circulation. In comparison, the low-metastatic HepG2 cells possess noticeably slower depletion kinetics. <40% HepG2 cells are depleted within the first hour. By 12 hours, ~55% HepG2 cells are depleted from the circulation.

4. Discussions and conclusions

The in-vivo-flow-cytometry is particularly useful for studying small animal models of tumors. For example, it is often difficult to obtain sufficient blood volume from a single mouse, whose total blood volume is only ~2 ml. This is particularly true when multiple blood samples are needed. With this in vivo technique, multiple measurements can be performed on a single animal over time, minimizing the number of animals and also eliminating variation between individuals.

Detection of green fluorescence signal on circulating tumor cells could be technically more difficult. It is particularly true for detection through blood because of the strong absorption below ~590 nm [15, 29]. Both excitation and emission will be attenuated respectively on the way in and on the way out. It might be a potential problem for in vivo flow cytometer to pick up the green fluorescence signal on circulating cells, depending on the green fluorescence level, although we have detected green fluorescence signal from different cell population in circulation (unpublished results). One method to enhance the sensitivity is to utilize the multi-photon detection, although with significantly higher cost. Low et al. developed a multiphoton intravital flow cytometer to quantify rare circulating tumor cells in vivo and achieved moderately higher sensitivity than confocal detection used in our current in-vivo-flow-cytometer [15, 16, 23, 29-31]. Another method is to use the photothermal/photoacoustic detection, which also improves the detection depth. Tuchin et al. developed a photothermal image flow cytometer to detect target cells in blood and lymph flow in vivo...
[20, 32, 33]. They used nanoparticles to enrich the rare cells and achieved significantly higher sensitivity [18, 19, 34].

The study here focuses on measuring circulating liver tumor cells. However the methods developed here shall be applicable to various potential biomarkers in circulation for detecting cancer, characterizing pathologic malignant tumors, assessing disease prognosis, and for predicting and measuring response to treatments, thus providing invaluable information about detecting and treating cancer. Furthermore, the developed methods here will be useful to monitor the circulating cells in general.

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