Label-free detection of circulating melanoma cells by in vivo photoacoustic flow cytometry

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ABSTRACT

Melanoma is a malignant tumor of melanocytes. Melanoma cells have high light absorption due to melanin highly contained in melanoma cells. This property is employed for the detection of circulating melanoma cell by in vivo photoacoustic flow cytometry (PAFC), which is based on photoacoustic effect. Compared to in vivo flow cytometry based on fluorescence, PAFC can employ high melanin content of melanoma cells as endogenous biomarkers to detect circulating melanoma cells in vivo. We have developed in vitro experiments to prove the ability of PAFC system of detecting photoacoustic signals from melanoma cells. For in vivo experiments, we have constructed a model of melanoma tumor bearing mice by inoculating highly metastatic murine melanoma cancer cells, B16F10 with subcutaneous injection. PA signals are detected in the blood vessels of mouse ears in vivo. The raw signal detected from target cells often contains some noise caused by electronic devices, such as background noise and thermal noise. We choose the Wavelet denoising method to effectively distinguish the target signal from background noise. Processing in time domain and frequency domain would be combined to analyze the signal after denoising. This algorithm contains time domain filter and frequency transformation. The frequency spectrum image of the signal contains distinctive features that can be used to analyze the property of target cells or particles. The processing methods have a great potential for analyzing signals accurately and rapidly. By counting circulating melanoma cells termly, we obtain the number variation of circulating melanoma cells as melanoma metastasized. Those results show that PAFC is a noninvasive and label-free method to detect melanoma metastases in blood or lymph circulation.

Keywords: in vivo photoacoustic flow cytometry, circulating tumor cells, melanoma, B16F10 cell

1. INTRODUCTION

Melanoma is a kind of skin cancer forming from melanocytes (pigment-containing cells in the skin) \textsuperscript{1}. People living in sunny climates are more likely to develop melanoma. It indicates ultraviolet light (UV) exposure \textsuperscript{2} is the primary cause, which is in conjunction with the amount of skin pigmentation in the population \textsuperscript{3}. The incidence rates of melanoma are expected to be comparable to breast cancer diagnosed in the US in 2014, and are still increasing \textsuperscript{4}. Melanoma has been one of the three most common cancers among males \textsuperscript{5} and accounts for about 80\% of death in all skin cancers \textsuperscript{6}, indicating that melanoma brings a great threat to human lives. While if melanoma is diagnosed and treated at an early stage before it builds new tumors in other sites or only a small number of circulating tumor cells (CTCs) emerge in the blood circulation, the cure rate will improve greatly.

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Metastatic tumors are very common in the late stages of cancer. New metastasizing tumors always occur in lungs, liver, brain, and bones via the blood or the lymphatics or through both routes. As tumors especially malignant tumors (cancer) develop, they can create new vessels to get nutrition and give cancer cells lots of chances to detach original sites and transport to new sites. Some of the detached malignant cells will acquire the ability to penetrate the walls of lymphatic and/or blood vessels, after which they are able to circulate through the blood stream to other sites and tissues in the body. Circulating tumor cells will fight against the body’s defense system to reattach themselves in a new site and form a newly detectable tumor which is called a metastatic (or secondary) tumor. Though less than 1 in 10,000 circulating tumor cells can survive in the circulation system to create a new tumor, the circulation of blood can take them everywhere. If cancer metastasizes to other tissues or organs, it usually dramatically decreases a patient’s likelihood of survival. Circulating tumor cell counting has been shown as a prognostic marker for metastasis development. Early detection, when circulating tumor cells in circulation system has been shown as a prognostic marker for metastasis development. Early detection, when circulating tumor cells in circulation system are rare, is of great importance to prolong life-span or even cure cancers.

Conventional flow cytometry is a laser-based technique by suspending cells in a stream of fluid. It is usually employed in cell counting, cell sorting, biomarker detection and protein engineering. When the suspended cells pass through the sheath, scattering and fluorescent light can be detected. It is routinely used in the diagnosis of health disorders, especially blood cancers. Through circulating tumor cell counting in the blood by flow cytometry, cancer metastases, recurrence and therapeutic efficacy can be assessed efficiently. Conventional flow cytometry needs invasive blood extraction in living organisms. The invasive extraction may introduce artifacts and make it not possible to conduct long-term monitoring of the cells in the complex natural environment. Meanwhile, the small blood volume (5-10 mL) is another limitation. Due to these limitations, In vivo flow cytometry (IVFC) based on fluorescence is developed for circulating tumor cells detection in vivo using vessels as natural liquid sheaths and overcoming the blood volume limitation. However, only cells labeled with fluorescent dyes could be detected by In vivo flow cytometry. The toxicity from the fluorescent dyes may affect the cell properties. Thus, photoacoustic flow cytometry, based on the photoacoustic effect, is developed, which might be a label-free method for some special cancer detection such as melanoma detection. Photoacoustic flow cytometry is first realized by Zharov et al. When a laser irradiates biological tissues, the tissues absorb the energy while a sound or stress wave is produced because of the thermoelastic expansion induced by a slight temperature rise. Depending on this detection principle, noninvasive and label-free detection of melanoma cells can be realized. In vivo flow cytometry has a number of applications such as in vivo detection of circulating tumor cells, nanoparticles, pathogens.

Here, we have developed a PAFC system to realize noninvasive and label-free detection of circulating melanoma cells. B16F10, a high metastatic type of melanoma, has a high light absorption due to its high content of melanin which can serve as a natural marker of melanoma circulating tumor cells detection. Photoacoustic signals from B16F10 are much higher than background signals and contribute to signal detection. It is necessary to analyze the signals to obtain more information of the cells. No matter where the photoacoustic signals come from, tumor cells, red blood cells or tissues like blood vessel, skin and hair, the methods of signal processing are similar. Here, we analyze the signals from human hair to explain the methods.

2. MATERIALS AND METHODS

2.1 Cell culture and animal model

B16F10 (a murine high metastatic melanoma cancer cell line) was directly bought from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai Institute of Cell Biology). The B16F10 cells were cultured according to standard procedures, including serial passage in high-glucose DMEM medium (GIBCO) with 10% fetal bovine serum (HyClone). Cells were incubated at 37°C with 5% CO2 in the incubator.

The experiments involved a tumor bearing nude model. Five male BALB/c nude mice (Shanghai SLAC Laboratory Animal Co., 20 ± 2 g, 6-8 week) were prepared for in vivo detection of melanoma CTCs. In order to construct melanoma bearing mice, five mice were inoculated of melanoma cells at their right flanks by subcutaneous injection of 10⁶ B16F10 cells per mouse.
2.2 Experimental setup

PAFC system designed for melanoma CTCs detection was excited by an Nd: YAG laser (wavelength: 1064 nm, pulse width: 2 ns, repetition rate: 5000 Hz) which was then reshaped by a cylindrical lens and focused by an objective (40X, NA=0.6) to the specimen. The laser focus spot was a narrow slit, perpendicular to the direction of blood flow and covering the full width of an appropriate ear artery to ensure no cells missed from excitation. The image was recorded by a CCD camera. An ultrasound transducer (central frequency: 10 MHz, element diameter: 3 mm) was used to detect PA signals. The whole setup was illustrated in Figure 1.

For in vitro experiments, melanoma cells were suspended in PBS liquid. We put the liquid in a syringe which connected a capillary tube. The liquid in the syringe was pulled by a syringe pump. For in vivo detection, after standard anesthesia (pentobarbital sodium, 20 mL/20g, concentration: 10 mg/mL), the nude mice were placed on the sample stage with a heated board to keep body temperature. Warm water was used to couple the ultrasound transducer with the tissue. We detected CTCs at the end of each week after B16F10 cell inoculation to monitor the melanoma metastases. This study was approved by the Ethical Committee of Animal Experiments of Med-X Research Institute and School of Biomedical Engineering, Shanghai Jiao Tong University.

Figure 1. PAFC system is designed mainly in three parts. PA signal excitation part includes a laser, two reflectors (R), a cylindrical lens, a mechanical slit, an achromatic lens, an optical filter and an objective. Imaging navigation part includes an LED light, a reflector, a focus lens, a filter, and a CCD camera. PA signal detection part includes an ultrasound transducer, a pre-amplifier, a DAQ card and a computer. The laser and DAQ card are triggered by a function generator synchronously. The image is recorded by an image acquisition card. The upper right picture shows PA signals from melanoma cells in vitro. The bottom right picture shows an image of capillaries in a mouse ear.

3. RESULTS AND DISCUSSIONS

For in vitro experiments, we suspended melanoma cells in PBS and pulled them to flow in a capillary tube to mimic a blood vessel. The detected photoacoustic signals were shown in Figure 2. It was obvious that photoacoustic signals from melanoma cells were particularly higher than the background signals after signal denoising and averaging. The results
proved our PAFC system was capable to detect melanoma cells signals. For in vivo experiments, five mice inoculated with B16F10 cells were detected on ear capillaries (typically arteries). Clear images of appropriate ear vessels after anesthesia can be seen in Figure 1 (bottom right). When melanin-contained melanoma CTCs flowed through the laser spot across the target vessels, photoacoustic signals were excited and detected by PAFC system. Processed by averaging and denoising algorithm (Figure 2), melanoma CTCs signals could be well distinguished after the signal noise ratio (SNR) was improved efficiently. Though flow velocity of the blood was high, the high sampling rate of 50 MHz made sure to fulfill measurement of fast-flowing cells.

In our experiments, the life-span of each mouse was recorded. After B16F10 inoculation, the five mice started to die from the 26th day after B16F10 inoculation and suffered gradual death during the next few days. It indicated that B16F10 deadly threatened nude mouse's life.

After signal denoising, we focused on time domain analysis and frequency domain analysis. Target cells or other tissues were irradiated by a focused laser beam while the signals were detected by an ultrasound transducer attached to the sample. In a measurement period, targeting photoacoustic signals from target cells or tissues correspond with the peaks in the whole signal. This phenomenon allows us to analyze the photoacoustic signal through finding signal peaks. The amplitude and number of peaks could reflect the constitute of target cells and the concentration of cells in blood or lymph flow. More absorption in cell would lead to higher peak. In time domain analysis, we can set threshold values and make comparison between threshold values and peak values in the signal. When the signal exceeding the threshold values appears, it means that we have detected the target cells. By analyzing and processing the signal coming from known target cells in time and frequency domain, we can identify cell-specific properties. Using those properties, while no prior knowledge of the cell parameters is required, the type of the cell measured can be inferred using the time domain and frequency domain analysis methods together to analyze the measured photoacoustic signal.

We recorded the melanoma CTC numbers of the five melanoma tumor bearing mice by PAFC once a week after B16F10 inoculation. PAFC detection of B16F10 CTCs on appropriate vessels of mouse ears in vivo was 10 minute duration each time. Due to the death of the mice, we just recorded the data of the first four weeks after B16F10 inoculation. There were no more than 3 CTCs detected in the first week after B16F10 inoculation. However, the number increased dramatically in the following weeks.

![Figure 2. PA signals detected from melanoma in vitro. The upper signals are the original PA signals from melanoma cells in vitro, the bottom signals are the processed results of the upper signals by averaging 10 times and denoising algorithm with Matlab software.](http://proceedings.spiedigitallibrary.org/ on 03/25/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx)
In the second week after B16F10 inoculation, the detected CTC number in melanoma tumor bearing mice increased to approximately five cells per 10 minutes. In the third week, the detected CTC number in melanoma tumor bearing mice increased to approximately eleven cells per 10 minutes. In the fourth week, the detected CTC number in melanoma tumor bearing mice increased to approximately twenty-one cells per 10 minutes. Most of the melanoma tumor bearing mice become very sick after the fourth week and died.

By *in vitro* and *in vivo* experiments, we detected PA signals from melanoma CTCs and recorded the changes of melanoma CTC numbers in the process of melanoma metastases. The results indicated that our PAFC system had the ability to monitor melanoma CTCs during the melanoma metastases.

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