**In vivo** cell characteristic extraction and identification by photoacoustic flow cytography

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**Abstract:** We present a photoacoustic flow cytography with fast cross-sectional (B-scan) imaging to precisely identify specific cells in vivo. The B-scan imaging speed of the system is up to 200 frame/s with a lateral resolution of 1.5 μm, which allows to dynamically image the flowing cells within the microvascular. The shape, size and photoacoustic intensity of the target cells are extracted from streaming images and integrated into a standard pattern to distinguish cell types. Circulating red blood cells and melanoma cells in blood vessels are simultaneously identified on melanoma-bearing mouse model. The results demonstrate that in vivo photoacoustic flow cytography can provide cells characteristics analysis and cell type’s visual identification, which will be applied for noninvasively monitoring circulating tumor cells (CTCs) and analyzing hematologic diseases.

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**References and links**


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sorting labeled biological samples is known as a well-established technique for cell counting and cancer recurrence [1, 2]. Flow cytometry (FC) based on the detection of fluorescence of dye-analysis. Due to the one-to-one relationship between PA image and the sample absorption technique capable of accurately identifying cell types is desiderated in the field of cell signal excited by a linear configuration laser beam. On account of cells aggregation and monitor melanoma CTCs [9–12]. However, the PAFC is based on analyzing the overall PA attenuation in biological media. PAFC can be applied to developed to overcome these shortcomings [7, 8]. It's needless to take optical background undesirable efficiency in labeling targeted cells with fluorescent-dye.

Metastases is known as a process that circulating tumor cells (CTCs) separate from the primary tumor and move to other organs through the blood, which is the leading cause of deaths from cancer. Hence, CTCs in the blood have become a marker of indicating metastasis, cancer recurrence [1, 2]. Flow cytometry (FC) based on the detection of fluorescence of dye-labeled biological samples is known as a well-established technique for cell counting and sorting in vitro [3, 4], and it has been developed to in vivo monitor CTCs with high-throughput screening of large blood volumes [4–6]. However, the applications of in vivo FC are restricted by strong autofluorescence background, high light scattering, as well as undesirable efficiency in labeling targeted cells with fluorescent-dye.

Photoacoustic flow cytometry (PAFC) based on photoacoustic (PA) effect has been Photoacoustic flow cytometry (PAFC) based on photoacoustic (PA) effect has been introduced to overcome these shortcomings [7, 8]. It’s needless to take optical background into consideration, since the PAFC technology is stimulated by the light and detects emitted PA signals. And acoustic waves with much longer wavelengths, are resistant to scattering and attenuation in biological media. PAFC can be applied to in vivo, label-free, and noninvasive monitor melanoma CTCs [9–12]. However, the PAFC is based on analyzing the overall PA signal excited by a linear configuration laser beam. On account of cells aggregation and various absorbers under the illumination, the difficulty in distinguishing active PA signals may easily lead to misidentification and false negatives of cells [11, 12]. An improved technique capable of accurately identifying cell types is desiderated in the field of cell analysis. Due to the one-to-one relationship between PA image and the sample absorption

1. Introduction

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Photoacoustic flow cytometry (PAFC) based on photoacoustic (PA) effect has been developed to overcome these shortcomings [7, 8]. It’s needless to take optical background into consideration, since the PAFC technology is stimulated by the light and detects emitted PA signals. And acoustic waves with much longer wavelengths, are resistant to scattering and attenuation in biological media. PAFC can be applied to in vivo, label-free, and noninvasive monitor melanoma CTCs [9–12]. However, the PAFC is based on analyzing the overall PA signal excited by a linear configuration laser beam. On account of cells aggregation and various absorbers under the illumination, the difficulty in distinguishing active PA signals may easily lead to misidentification and false negatives of cells [11, 12]. An improved technique capable of accurately identifying cell types is desiderated in the field of cell analysis. Due to the one-to-one relationship between PA image and the sample absorption...
distribution [13, 14], fast cross-sectional (B-scan) PA imaging with high-resolution can display a variety of \textit{in vivo} absorbing cells that flowing through the blood vessel [15, 16]. And identification of cell types based on image analysis can be of higher accuracy. This is because the image not only visualizes microscopic cells, but also offers enough cell property information for recognition, such as shape, size and intensity distribution. Based on this thoughtfulness, we present an \textit{in vivo} photoacoustic flow cytography to precisely identify and count specific cells in this paper. The system is equipped with a two-dimensional scanning galvanometer to obtain high-speed B-scan imaging. The lateral resolution is measured to be 1.5 $\mu$m by imaging a sharp edge. Cells characteristics including shape, size as well as PA intensity are extracted from PA images, and integrate into a standard pattern to distinguish cell types. Then the \textit{in vivo} identification ability of photoacoustic flow cytography is testified by identifying and counting melanoma CTCs and red blood cells (RBCs) in blood vessels on melanoma-bearing mice. Herein, the system discerns \textit{in vivo} cell types by their intrinsic properties in graphs rather than analysis of indistinguishable signals [17–19], which achieves a great advancement in precision and visualization.

2. Experimental setup and materials

The schematic of the system is shown in Fig. 1(a). A microchip laser (HLX-I-F005, Horus Laser), operating 8 ns pulse width at 532 nm with a 30 KHz repetition rate, was used as the irradiation source, which provides enough contrast of RBCs and melanoma cells in PA imaging. The irradiation pulses pass through a two-dimensional (2-D) scanning galvanometer (6231H; Cambridge Technology, Inc., U.S.), scanning lens and tube lens, then are focused by the long working distance plan objective (LWDPO) and irradiated the test sample to produce PA signals. The intensity and stability of the laser beam are monitored and calibrated by a photodiode. The numerical aperture (NA) of LWDPO is 0.55 and working distance is 7 mm. The signals are detected by a custom-made hollow focused ultrasonic transducer with a 10 MHz center frequency. Through an amplifier (ZFL-500, Minicircuits) and a dual-channel data acquisition card (NI5124, National Instruments Corp., USA), PA signals are recorded in the computer for reconstructing 2-D images by the maximum amplitude projection along the z axis. A CCD camera (MD30, Mshot Co. Ltd, China) is installed to obtain optical pictures of test samples.

The lateral resolution of the system was measured by imaging a sharp-edged surgical blade (Fig. 1(b)). The full width at half-maximum (FWHM) of the line spread function was estimated to be 1.5 $\mu$m (Fig. 1(c)). The axial resolution of the system was estimated to be $\sim$132 $\mu$m by the system bandwidth and the speed of acoustic wave in tissue. Although the B-scan rate of the photoacoustic flow cytography currently was limited by the scanning galvanometer frequency of 5 kHz, the B-scan rate could be capable of 200 frames/s in a small scanning range with high lateral resolution. When operated in a repetitive B-scan mode, the system was allowed to real-time dynamically image of flowing cells passing through blood vessels.

In the experiments, murine melanoma B16F10 cells with high metastases (JRDUN Biotechnology Co. Ltd) were cultured in dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), and incubated in a suitable incubator with a constant temperature of 37°C under 5% CO$_2$ in air atmosphere. In the \textit{in vitro} study, blood containing anticoagulant was derived from the mouse. The data of \textit{in vivo} study were acquired on BALB/c mouse (age: 6–8 weeks; bodyweight: 30-32 g), which were obtained from Center of Experimental Animals at SunYat-sen University. Animal procedures were carried out in accordance with the laboratory animal protocols approved by the South China Normal University. The melanoma-bearing mice models were achieved by subcutaneous inoculation of melanoma cells, and the concentration of injected melanoma cells is about $1 \times 10^7$/100 uL per mouse. The hairs of the mouse ear was chemically removed before imaging and the
mouse were intraperitoneally anesthetized (sodium pentobarbital, 40 mg/kg; supplemental, 10 mg/kg/h) to keep the mouse without movement during the experiment.

3. Results

3.1. Light absorption spectra and PA signal intensity of blood components

To verify that PA signal intensity can be used as a character for identifying specific cells, we measured the optical absorption spectra and the corresponding PA signals intensities of blood.
components using a multifunctional microplate reader (ELISA, Infinite 200, TECAN, Switzerland) and a PA microscopy respectively. In the experiment, metastatic melanoma cells in circulation were imitated using B16F10 cells detached from culture dishes. Blood cells were separated from mouse whole blood, and then suspended in phosphate buffer saline (PBS). Finally, the number particles of RBCs, the mixture of white blood cells (WBCs) and platelet (PLT), and melanoma cells suspended in 100 μL saline solution were about the same, ~1.35 × 10^7, ~0.9 × 10^7 and ~1.25 × 10^7, respectively. As shown in Fig. 2(a), RBCs reached its absorption peaks at 420 nm, 550 nm and 580 nm. The absorption of melanoma cells was higher than that of RBCs ranged from 440 nm to 750 nm. As the 532 nm laser beam illumination, both RBCs and melanoma cells were found to generate remarkable PA signals indicated in Fig. 2(b). The absorption of plasma, the mixture of WBCs and platelets, and PBS were relatively low, producing negligible PA signals that were almost submerged in the background noise. Meanwhile, under the same energy, the PA intensity of melanoma cells was higher (1.2-2.5 times) than that of RBCs at 532 nm. Thus, the PA signal intensity can be extracted as a parameter for recognizing RBCs and melanoma cells.

3.2. Morphology comparison of RBCs and B16F10 cells

To validate that cell diameter is another characteristic to discern RBCs and melanoma cells, RBCs, adherent melanoma cell, and suspended melanoma cell in RBCs were measured in vitro, as shown in Fig. 3(a), 3(b) and 3(c) respectively. RBCs were 6-7 μm in diameter, and suspended melanoma cells were larger, 10-20 μm in diameter, which were consistent with previous work [11, 12]. The adherent melanoma cells grown on glass were visible highlighted, that’s because abundant melanin in cells lead to higher PA signals. Also, some exocytosis particles parcelled melanin were visible around the melanoma cell in PA image (Fig. 3(b)) and optical image (Fig. 3(e)). Figure 3(c) and Fig. 3(f) were images obtained on the focal plane of melanoma cells. The growth of suspended melanoma cells became stunted after away from culture medium, and their melanin content was relatively low. The imaging results showed that suspended RBCs and melanoma cells were approximately round in shape but they had evident differences in diameter. Therefore, diameter can be extracted as another characteristic to identify cell types.

![Fig. 3. Morphology comparison. (a), (b) and (c) PA images of RBCs, adherent melanoma B16F10 cells, and suspended B16F10 cells in RBCs. The scanning interval was 1 μm. (d), (e) and (f) The corresponding optical microscope images.](image-url)
3.3. Integration and verification of characteristic patterns

Pattern recognition [20–24] refers to the use of known characteristics of a target cell as a template and detection of its related similar sets of characteristics in a usually larger cell image. Assessment of these similarities requires some judgments that correlation operators can be chosen to distinguish whether the pattern of characteristics matches. Therefore, given characteristics of a target cell formed a pattern \( n \), the larger field image \( s \), the maximum correlation peaks between \( n \) and \( s \), \( R(n, s) = n \ast s \) is the identification of its instances. The statistical methods we used here were Bayesian statistics [22, 23]. Meanwhile, different types of cells have different diameters and light absorption intensity, both of these parameters has a range of variations for each cell types. Thus, before attempting pattern recognition, integration and verification of characteristic patterns of target cells should be carried out.

![Fig. 4. Characteristic extraction and identification. (a) Processes of obtaining cell diameters after edge detection, filling holes and extraction. \( D_l \) and \( D_s \) were the long and short diameters. (b) The maximum PA signal intensity extracted from a B16F10 cell image. The bar shown in figures represents 3 \( \mu m \). (c) Scatter plot of the long versus short diameters. (d) Scatter plot of the long diameter and PA intensity. (e) Scatter plot of the \( \Gamma \) parameters of cells. The hollow circles are RBCs and the filled circles are B16F10 cells.](image)

In order to secure the optimal standard pattern for recognition, photoacoustic flow cytography with different characteristics patterns was used to recognize 50 standard RBCs and 50 standard B16F10 cells \textit{in vitro}. Cell diameters were obtained after images processes [21] of edge detection, filling holes and extraction (Fig. 4(a)). And the maximum PA intensity was extracted from PA image of the standard cell. Figure 4(b) showed the extractive processes of the maximum PA intensity of a melanoma cell. By characteristics of the long and short cell diameters, identification analysis was performed (Fig. 4(c)). There existed a small intersection of diameters between these two cells, labeled with red solid circle, which easily caused inherent misjudgments. In consideration of the PA signal intensity versus the long diameter, four areas of interest were distinguished for the scatter plot in Fig. 4(d): I) cells with small diameters and low PA intensity, which were considered to be RBCs. III) cells with large diameters and high PA intensity considered to be B16F10 cells. II) and IV) areas with at least 8% uncertainty, cell types were undetermined. To reduce the uncertainty, characteristics to identify cell types were further optimized. Another parameter \( \Gamma \) (defined as \( \Gamma = P \times D \), where \( P \) is the maximum intensity of the measured cell, \( D \) is the cell’s long diameter) was integrated by the maximum PA intensity and the cell’s long diameter into pattern for identification.
These cells shown in Fig. 4(e) were divided into three groups. The $\Gamma$ values higher than the solid line were indicative of B16F10 cells. And the $\Gamma$ values of RBCs were considered between the solid line and the dashed line. The points under the dashed line indicated other cells or the background noise. These identification results were conformed to the cell groups and it became obvious that the detection accuracy of photoacoustic flow cytography with the $\Gamma$ characteristic has improved considerably comparing to other characters identification. Therefore, the $\Gamma$ parameter in PA imaging can be used as the optimal standard pattern to recognize cell types.

3.4. In vivo, imaging and identification of RBCs and melanoma cells

To testify the in vivo cells identification ability of photoacoustic flow cytography with the optimal standard pattern, experiments were performed on the superficial blood vessels in the mice ear. Firstly, a microvasculature image of an area at a mouse ear edge (Fig. 5(a)) was scanned with a step of 2 $\mu$m. Then two appropriate blood vessels were successively chosen for obtaining measurements, which had diameters of 10 $\mu$m and 18 $\mu$m. After this, the system worked in the fast repetitive B-scan mode. The scanning lines, labeled x1 and x2, were perpendicular to the selected blood vessels and were set to 25 $\mu$m in 20 steps. The B-scan time series cumulating images were shown in Fig. 5(b) at one point. In x1-t image, RBCs were circular and flowed one after another. And in x2-t image, RBCs appeared connected to each other. These two white dashed lines represented walls of the virtual vessel. Herein, the system with pattern recognition could recognize 8 and 15 RBCs respectively (Fig. 5(b)), and cells in white squares were consistent with RBC pattern. However those pointed by yellow arrows might be other types of cells, because they cannot match the standard characteristic pattern. The results show that the system is feasible to in vivo image of flowing cells and identify RBCs.

![Figure 5](image_url)

Fig. 5. In vivo recognition of RBCs and melanoma CTCs. (a) PA image of a capillary at the edge of a mouse ear. (b) B-scan x-t image of cells in vessels located in (a). (c) Melanoma tumor growth in mouse dorsum. (d) The superficial microvasculature PA image in a mouse ear. (e) B-scan x-t image of cells in a selected vessel labeled x3 in (d). The diameters of selected vessels were 10 $\mu$m, 18 $\mu$m and 15 $\mu$m respectively. The white dotted lines represented walls of vessels. And the recognized melanoma CTCs and RBCs were labeled with yellow and white squares respectively. (f) The number of melanoma CTCs recorded in one minute as a function of weeks after tumor inoculation.
For a further demonstration, photoacoustic flow cytography was used to simultaneously identify melanoma CTCs and RBCs in melanoma metastatic process on the melanoma-bearing mice. The melanoma primary tumor progression was shown in Fig. 5(c), and its melanoma levels were visibly higher during the second week after B16F10 cells inoculation. Using the same experimental steps on normal mice, a vessel with a 15 μm diameter was selected, and the system operated on the B-scan model, and handled identification on the B-scan time series cumulating images at 1 second intervals. It could be seen that, there were several relevant cells, one B16F10 cell and 13 RBCs, in a yellow square and white squares, respectively (Fig. 5(e)). The above recognition showed the possibility of the presence of melanoma cells in circulation. Then, the system was used to in vivo continuously identify and count CTCs on six melanoma-bearing mice to monitor metastatic process of melanoma. The development of melanoma cancer metastasis was reflected in Fig. 5(f), which were statistics of the average number of CTCs in one minute at the end of every week after tumor inoculation. There were about 0 CTCs/min at the end of first week after inoculation, and about 2.06 CTCs/min at the end of fifth week after inoculation. The results illustrate that the system is able to simultaneously discern RBCs and melanoma cells, and the number of CTCs can be used to assess melanoma metastasis development.

4. Discussions and conclusions

In this work, RBCs and melanoma cells are successfully identified and counted simultaneously in microvessel by an in vivo photoacoustic flow cytography system. This system works on fast repetitive B-scan model with optical resolution to in vivo acquire PA imaging of RBCs and melanoma cells. Automatically, pattern recognition at 1 s intervals is performed on the B-scan time series accumulating images to discern cell types. In comparison with PAFC, unlike distinguishing an overall signal of absorbers, photoacoustic flow cytography takes advantage of cells characteristics to identify and count flowing cells, which is a more accurate and reliable method. This method can be not only applied for CTCs detection to assess the development of metastasis, but also used for analyzing hematologic diseases. Sickle-cell anemia as one of hematologic diseases has an abnormal sickle-like shape of RBCs and an average volume of a red blood corpuscle below normal range [25, 26]. The photoacoustic flow cytography system with the cells properties including shape, size and light absorption intensity, can be a valid means to distinguish RBCs and calculate mean cell volume for label-free and noninvasively auxiliary diagnosis of the disease [11, 27–29].

It is worth noting that the B-scan rate of the system is up to 200 frames/s over a small scanning range of 25 μm in 20 steps, which enables the system to obtain measurements in mouse superficial ear specific blood vessels. These vessels should have the velocity less than 0.4 mm/s, and the diameters of the vessels are better to smaller than 15 μm [30]. Using the equation \( v = D/t \) (\( v \) is the velocity of blood vessels, \( D \) is the diameter of blood cells, \( t \) is the time of cells traveling through the scanning line), the speed of blood flow in microvessel can be estimated. There are about 0.15 mm/s, 0.3 mm/s, and 0.21 mm/s of the three vessels indicated in Fig. 5, which are all in the testable ranges. Meanwhile, dislocation correction and interpolation have been processed before performing recognition on the image. However, additional works are still needed to further mature this technique. Firstly, in order to measure higher speed blood flow and capture much minute images of cells, the B-scan velocity and scanning steps should be increased. And this can be solved by using a faster scanning galvanometer in the future. Secondly, further improvements in axial resolution can be obtained by using a high frequency hollow ultrasonic transducer to acquire apparent details in z axis, which may help the system to realize analysis of blood cells in three-dimensions. In addition, the irradiation source of the system is the 532 nm laser beam, which makes RBCs and melanoma cells in PA images with enough contrast. At this source, the system is successfully demonstrated the capability of simultaneously identifying two types of cells. But if only for the sake of detecting melanoma cells in blood, the use of excitation in near infrared
spectral range will be better for the operation of this system, since the stronger PA intensity generated by the melanin will result in a higher contrast in PA images. At last, Using laser diode or LED for photoacoustic flow cytometry will be more inexpensive, compact, fast and portable, which may be beneficial to the clinical application [31].

In conclusion, an in vivo photoacoustic flow cytography is demonstrated to identify specific cells. Using RBCs and melanoma cells for example, cell types are discerned by their intrinsic characteristics, including shape, size and PA intensity, which achieve a great advancement in precision and visualization. This technique may provide an effective way to monitor CTCs and analyzing hematologic diseases in vivo.

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