

## Photoacoustic detection of circulating melanoma cells in late stage patients

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Melanoma is the deadliest skin cancer and is responsible for over 7000 deaths in the US annually. The spread of cancer, or metastasis, is responsible for these deaths, as secondary tumors interrupt normal organ function. Circulating tumor cells, or those cells that spread throughout the body from the primary tumor, are thought to be responsible for metastasis. We developed an optical method, photoacoustic flow cytometry, in order to detect and enumerate circulating melanoma cells (CMCs) from blood samples of patients. We tested the blood of Stage IV melanoma patients to show the ability of the photoacoustic flow cytometer to detect these rare cells in blood. We then tested the system on archived blood samples from Stage III melanoma patients with known outcomes to determine if detection of CMCs can predict future metastasis. We detected between 0 and 66 CMCs in Stage IV patients. For the Stage III study, we found that of those samples with CMCs, two remained disease free and five developed metastasis. Of those without CMCs, six remained disease free and one developed metastasis. We believe that photoacoustic detection of CMCs provides valuable information for the prediction of metastasis and we postulate a system for more accurate prognosis.

*Keywords:* Diagnosis; metastasis; photoacoustics.

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## 1. Introduction

Melanoma is the most rapidly growing type of cancer in the US. Over the last century, melanoma incidence has more than doubled every 10 to 20 years so that the current lifetime risk of getting melanoma in the US is about 1 in 55.<sup>1,2</sup> There are over 600,000 melanoma patients in this country, all of whom have some risk of their cancer becoming metastatic, thus entering the deadliest phase of this disease.

Circulating tumor cells (CTCs) are those cells that originate from cancerous lesions and propagate through the blood and lymph systems. CTCs may provide prognostic information for advanced cancer patients and are believed to be the mechanism by which secondary tumors spread throughout the body.<sup>3,4</sup> CTCs have been studied for a variety of solid tumors, including breast, prostate, lung, melanoma, and other cancers, with increasing evidence that their detection can be used to monitor response to therapy, detect relapse, and give overall indications of disease state.<sup>5-9</sup> Thus, capture and analysis of CTCs may provide valuable clinical information for management of cancer as well as provide avenues for basic research in cancer biology.<sup>10</sup>

Photoacoustics has been used for particle detection and counting to exploit optical absorption of materials of interest.<sup>11</sup> Photoacoustic flow cytometry, in which the medium containing cells of interest is under flow, has been used for detection of pigmented cells in body fluids.<sup>12,14</sup> In particular, Zharov *et al.*, performed *in vivo* detection of CTCs. This technique was further developed by Galanzha *et al.*, to use advanced signal processing to enhance the signal and account for skin pigmentation limitations.<sup>13</sup> The advantage of photoacoustics over conventional flow cytometry is that the laser induced ultrasonic response is only present in cells with natural pigmentation, such as melanoma, and can ignore other cells not relevant to disease diagnosis.

We have used an *in vitro* system for detecting and capturing CTCs,<sup>14-19</sup> that uses laser induced ultrasonic signals generated in cells of interest to indicate their presence in blood samples. Since our test is *in vitro*, we can capture the cells of interest for further analysis. Leukocytes in enriched blood samples lack a photoacoustic signature, so single CTCs can be detected among thousands or even millions of normal leukocytes extracted from whole

blood. After centrifugation to separate the buffy coat, we can perform photoacoustic detection and capture of targeted cells in less than 30 min.

### 1.1. CTC and photoacoustics background

Detection of metastatic disease is currently performed using imaging modalities, such as CT and MRI. These methods can only detect macroscopic tumors that may be too large or too invasive to be treated.<sup>20</sup> CTCs may indicate metastatic disease and have potential to provide valuable information for managing treatment.<sup>21</sup>

Much of the biology of CTCs is unknown due to the difficulty in detecting and isolating these rare cells. Researchers have found that the incidence of CTCs among normal blood cells is on the order of one in a billion,<sup>22</sup> thus their detection and isolation constitute a major engineering challenge. Only after CTCs can be quickly, reliably, and inexpensively captured can the fundamental questions about their biology be investigated. Of equal importance, monitoring CTCs may provide clinicians with diagnostic and prognostic information about their patients, possibly ushering a new era of therapy in which advanced cancer is fought cell by cell, rather than against large, macroscopic tumors, as is current practice.

We have developed the first photoacoustic flow cytometer for detecting CTCs (Fig. 1) and have implemented methods to capture these elusive cells for further study.<sup>14</sup> Photoacoustic detection offers a tremendous advantage for detecting pigmented cells, such as circulating melanoma cells (CMCs), since the native light absorber, *melanin*, is normally found within these cells. Most melanoma cells are highly pigmented, with estimates of amelanotic melanoma being less than 5%<sup>23</sup> or 1.8–8.1%, though this latter figure includes partially pigmented melanoma.<sup>24</sup> Using appropriate laser technology, we can selectively induce high frequency ultrasonic responses in CMCs, even when they are among millions of normal white blood cells.<sup>16,17</sup> Using photoacoustic detection along with microfluidic principles, we use our photoacoustic flow cytometer in which we capture single CMCs in the blood of melanoma patients. These CMCs are captured in suspension, unlike many CTC capture technologies in which cells are bound to a substrate. This

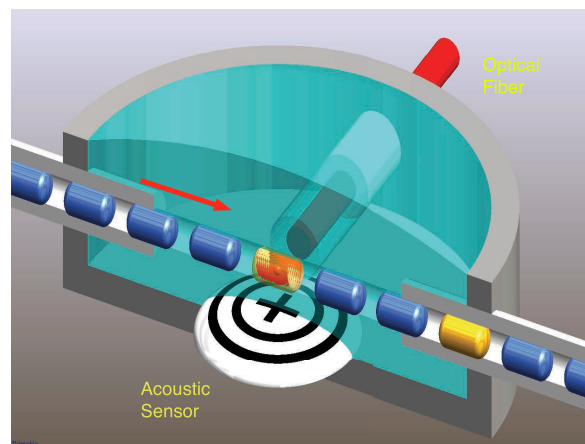


Fig. 1. The photoacoustic flow cytometer separates continuous flow of blood cells with air bubbles. The resulting blood cell suspension droplets are irradiated by laser light. Direction of flow of the droplets is indicated by the red arrow. Droplets that contain CTCs generate photoacoustic waves that are sensed by an acoustic transducer. These droplets are shunted off to a collection cuvette for further analysis. Bubbles that do not generate photoacoustic waves are assumed not to contain CTCs and are diverted for disposal.

advantage allows the CMC to exist in a natural, unaltered state, a distinct advantage when analyzing these cells. Early detection of melanoma is increasingly important, as new therapies are being developed. In March 2011, *ipilimumab* was approved by the FDA for treatment of late stage melanoma. In August of that year, *vemurafenib* was also approved. Additionally, many other drugs are in Phase III development. In contrast to the bleak history of melanoma therapy, the future holds promise for treatment of metastatic disease, increasing the need for screening, detection, and monitoring technologies.

### 1.2. Definition and alternate methods of detecting CTCs

The last decade has seen a vast increase in CTC detection research that includes a diverse set of technologies for detecting and capturing these rare cells. These technologies can primarily be categorized into five distinct groups: (1) molecular assays,<sup>25</sup> (2) immunohistochemical separation,<sup>27</sup> (3) microfluidics and filtration,<sup>28,29</sup> (4) flow cytometry,<sup>30</sup> and (5) separation by dielectric or ultrasonic properties.<sup>31,32</sup> Many of these technologies are developing rapidly. The CellSearch<sup>®</sup> system, which uses immunomagnetic separation, has achieved

FDA clearance for enumeration and has become the *de facto* standard for enumeration of CTCs that express EpCAM, which include most carcinomas.

Microfluidic technologies are compelling advances that include methods where whole blood is washed along chips against microposts with EpCAM antibodies.<sup>33</sup> Other ideas include microfluidic methods where capture is achieved by polymer grafted silicon nanostructures.<sup>34</sup> This idea has been conceptualized as a nanovelcro that specifically targets CTCs.

Some of the most advanced technologies for CTC detection include flow cytometry. Flow cytometry has been used in the clinic for leukemia and lymphoma, where millions of leukocytes are scanned for pathological cells.<sup>35</sup> Flow cytometry is particularly suited to these liquid tumors, since there is a relatively large population of leukemia cells, allowing robust statistical classifiers to be used.

Photoacoustic flow cytometry is a type of flow cytometry, though the use of laser induced ultrasonic waves in rare pigmented cells among thousands of nonabsorbing leukocytes in an enriched blood sample gives it the ability to find CMCs in human blood. The advantages of photoacoustic flow cytometry for capturing CMCs are: (1) CMCs remain in suspension, (2) the process is label free, (3) the process is fully automated, (4) recovery of CMCs is estimated to be 70% from whole blood, and (5) the process is rapid; a 5 ml blood sample can be processed in less than 30 min after initial enrichment.

## 2. Materials and Methods

Our photoacoustic flow cytometer is a laser based system for detecting, enumerating, and capturing pathological analytes in body fluids. As is the case for CMCs in enriched blood samples, it is sensitive for detecting pigmented particles under flow among nonabsorbing particles. Photoacoustic flow cytometry is particularly suited to detecting naturally pigmented cells, such as most melanoma cells, though nonpigmented cells, such as circulating breast cancer cells, can be labeled with light absorbing nanoparticles using antibody targeting. The testing paradigm is shown in Fig. 2. Whole blood is drawn from a human patient into a tube with anticoagulant. The blood is centrifuged using standard separation protocols to yield a buffy coat

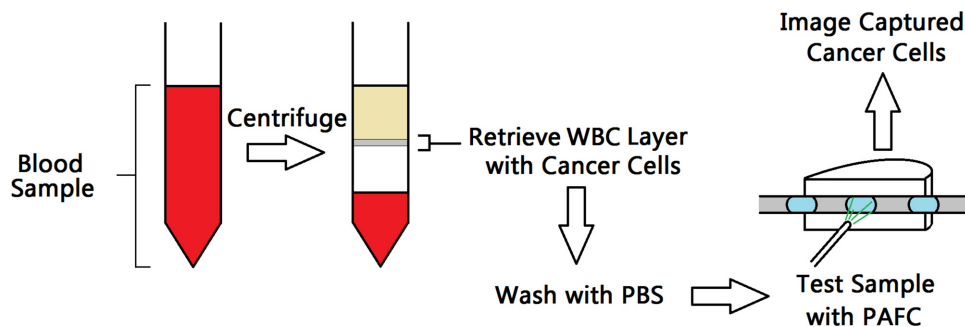


Fig. 2. Samples are centrifuged in order to separate blood into its components. The buffy coat, containing leukocytes and possibly CMCs, is taken and processed in the photoacoustic flow cytometer (PAFC).

consisting of white blood cells (WBCs) and any CTCs. CTCs settle in the buffy coat due to similar density to WBCs. The buffy coat is extracted, washed and introduced into the photoacoustic flow cytometer. Photoacoustic waves indicate the presence of CTCs, where they are extracted for imaging and other testing.

The photoacoustic flow cytometer uses a frequency tripled NdYAG laser pumping an optical parametric oscillator (Vibrant, Oportek, Carlsbad, California) tuned to 532 nm with a 5 ns pulse to irradiate cell suspensions under flow passing through a custom flow chamber. The laser light, at about 2 mJ per pulse, is ideal for inducing photoacoustic waves in melanoma cells. The repetition rate for the laser is 20 Hz. This light is coupled via a 1000 micron optical fiber with a numerical aperture of 0.22 (Thorlabs, Newton, New Jersey) and is delivered to the detection chamber (Fig. 1). The distance from the optical fiber to the flow chamber is approximately 10 mm, giving a fluence of about 8 mJ/cm<sup>2</sup>. This value assumes a uniform beam and 2 mJ per pulse, though pulse energy typically varies by about 5%.

Rather than irradiating a continuously flowing cell suspension, we induce two phase flow to facilitate cell capture. Two phase flow occurs when two immiscible liquids are injected into a flow system while maintaining a capillary number less than 0.01.<sup>36</sup> Steijn *et al.* developed a model that showed that only flow rates of the two fluids and the junction shape determined the droplet sizes. Using these principles, we are able to separate blood cell suspensions between air droplets. We use two syringe pumps (Harvard Apparatus, Holliston, Massachusetts) to pump the cell suspension and air. Thus, laser pulses irradiate droplets of cell suspension and those that produce photoacoustic waves

are captured for further analysis. Droplets not producing photoacoustic waves are assumed to contain only leukocytes and are released as waste. Each droplet was about 1  $\mu$ l.

Since we assume that the number of CMCs is small, since the cell suspension was 1 ml, using Poisson statistics, we assume that each droplet would have no CMCs, a few would have one CMC, and very few would have two or more. In the context of CMC in droplets, Poisson statistics can be stated as

$$P(k) = e^{-\lambda} \frac{\lambda^k}{k!}, \quad (1)$$

where  $P(k)$  is the probability of  $k$  CMCs in a droplet and  $\lambda$  is the expected value of CMCs per droplet. Since the suspension was well mixed, we believe the assumptions of the Poisson model to be valid. Even if there is some violation in the assumptions, the results are so skewed to having no or one CMC per droplet, that we believe the model to be applicable. That is, the assumption that in a well mixed suspension of cells divided into a thousand droplets, most droplets would have no CMCs, a few would have one, and almost none would have more.

For example, assuming 10 CMCs in 1 ml, Poisson statistics predicts over 99% would have no CMCs, almost a percent would have one CMC, and about  $5 \times 10^{-5}$  would have two or more. Thus, when we run the photoacoustic flow cytometer, droplets that had no photoacoustic waveforms were assumed to have no CMCs and those that generated a photoacoustic waveform were assumed to have one CMC. We measured the root mean square of the noise of this system by irradiating distilled water. We used an *ad hoc* threshold of twice the noise level to signify a photoacoustic waveform generated in a CMC.

## 2.1. Testing on stages III and IV melanoma patients

After extensive testing of the photoacoustic flow cytometer on cultured melanoma cells in PBS and spiked in blood samples<sup>14,17,19</sup> we tested clinical samples from melanoma patients. The first set of testing was done on 37 Stage IV patients. These samples were 1 ml each. In order to provide a negative control, we tested 12 healthy human subjects, processing 1 ml of blood in the same manner.

In order to show the efficacy of the photoacoustic flow cytometer to predict metastasis, we tested archived samples from 14 Stage III melanoma patients. The patients were either disease free or became metastatic within two years.

## 2.2. Statistical analysis

The Poisson statistics information was computed using Mathematica (Wolfram Research, Champaign, Illinois). Fisher's Exact test was used to analyze the Stage III patient data, with the categories being CMCs detected or not. The observed states are being disease free or advancing to metastasis within two years. This information was determined from archival patient information. Fisher's Exact test was calculated using Matlab (Mathworks, Natick, Massachusetts).

## 3. Results

The results of the Stage IV patient samples are shown in Fig. 3, with six patients showing no CMCs

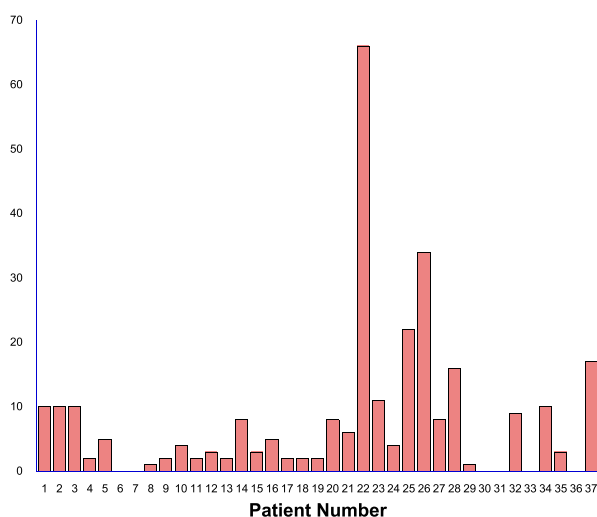


Fig. 3. Bar graph showing the number of CMCs detected in 37 Stage IV melanoma patients.

Table 1. Contingency table showing the numbers of patient samples that had CMCs and those that had none with respect to whether the patients remained disease free or became metastatic within two years.

	Disease free	Metastatic within two years
CMCs detected	2	5
CMCs not detected	6	1

and the others showing anywhere from two to 66 CMCs in the milliliter sample. Concurrent with this study, we tested healthy human subjects who showed no photoacoustic events, indicating no CMCs.

For the 14 Stage III patients, the results are shown in Table 1. Of the patients who remained disease free, six had no CMCs detected, while two did. Of those patients who became metastatic, five showed CMCs and one did not. Using Fisher's Exact test, these data gave a  $p$ -value of 0.10 for the two groups.

## 4. Discussion

The ability to exploit the optical differences between pigmented and nonpigmented cells, combined with the robust signal carrying ability of ultrasonic waves makes photoacoustic flow cytometry a valuable tool for detecting, counting, and capturing CMCs. We used this system to investigate the presence of CMCs in blood samples from human patients. The Stage IV study showed the ability to detect and enumerate the cells. In contrast, none of the healthy human subjects showed such photoacoustic responses. We believe that the photoacoustic waves generated within the buffy coat of melanoma patients are due to pigmented melanoma cells.

To verify the nature of the cells generating photoacoustic waves, we captured the positive droplets and stained them using standard immunohistochemical techniques. Figure 4 shows a brightfield image of captured cells, many of which are leukocytes. The second image shows a cell in the center fluorescing green, indicating the MART1 antigen, characteristic of melanoma cells. In separate work, we have shown that captured cells fluoresce from melanin granules after treatment with formalin.

We are planning more comprehensive study of photoacoustically captured CMCs and MART1, and perhaps SOX10, but the interpretation is



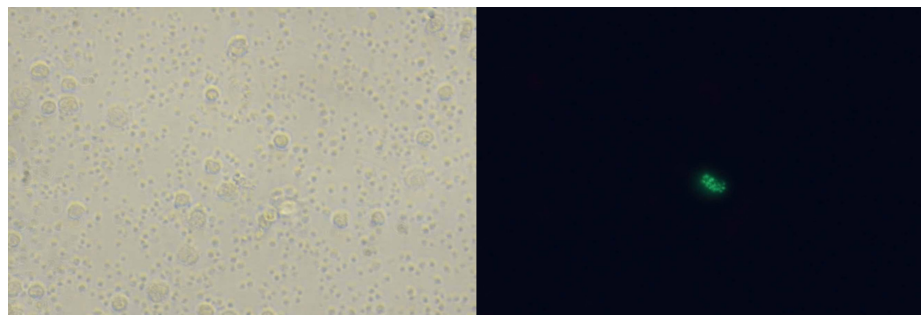


Fig. 4. (Left) Brightfield image of captured cells. (Right) Fluorescent image of the same field showing the presence of a melanoma cell with MART1 expression.

complicated by factors such as recovery rate of CMCs, immunohistochemical staining technique, and whether the particular cells express MART1 or SOX10. Though these are common surface markers in melanoma, the uncertainty of their expression may confound our results. In the meantime, we are developing a statistical model using information from serial samples from melanoma patients to provide more accurate prediction of metastasis.

The results of the fourteen Stage III patients give some indication that CMC numbers provides information about the development of metastasis. The contingency analysis gave a  $p$ -value of 0.10. While statistical significance is commonly determined by the arbitrary assignment of 0.05 as a significance level, this  $p$ -value shows some correlation between the detection of CMCs and the onset of metastasis. We believe that by testing serial samples from melanoma patients and comparing to a threshold number may give simple and accurate predictive value using photoacoustic flow cytometry. Also to improve accuracy, we have been investigating signal analysis, including the use of machine learning, to distinguish photoacoustic waves generated from CMCs from noise.

In any case, regardless of what is being detected, if the photoacoustic flow cytometer is considered a system acting upon an input, namely blood samples from Stage III melanoma patients, we have shown the system gives preliminary predictive value about the probability of advanced disease.

However, we must consider factors such as the vast and unknown biological variability of patients, their propensity to shed CMCs into blood, the single sample point, limited sample volume, and other variables. Ideally, the entire blood volume of a patient should be monitored continuously in order to minimize many of these variables as was done by

Galanzha *et al.*<sup>13</sup> However, a practical test, such as a small volume tested once or during subsequent clinical visits, is more realizable and more apt to be adopted and adds the ability to perform molecular characterization of these cells. While these limitations of the current testing are acknowledged, future work will focus on improving the overall predictive value of this method.

Research in CTC analysis continues to push the boundaries of cancer prediction and diagnosis. In particular, the significance of detecting CMCs and their relationship to developing metastatic disease is supported by other technologies.<sup>37</sup> Photoacoustic flow cytometry has powerful, unique capabilities to find rare CMCs in the blood of melanoma patients. We are continuing our studies to develop a predictive system for metastasis based on our Stage III data. Additionally, we are performing engineering improvements to increase throughput and purify capture volume. Based on our results thus far and our current projects, photoacoustic flow cytometry may be an effective, low cost method for detection and capture of CMCs, providing new information for monitoring disease state and clinical management of disease.

## Conflict of Interest

Martin E. Sanders and John A. Viator are officers in Acousys Biodevices Inc, a company formed to commercialize photoacoustic methods, and have equity in the company. The remaining authors have no competing financial interests.

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