

# Using In Vivo Photoacoustic Flow Cytometry to Assess Circulating Tumor Cells in Melanoma

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**Melanoma is widely metastatic at an early stage. There is an increasing need to develop equipment that can detect circulating tumor cells (CTCs) faster than current assays, which often detect metastasis after it has spread to a fatal level. Photoacoustic flow cytometry (PAFC) is based on nonradiative relaxation of absorbed laser energy into heat that produces an accompanying sound effect. The photoacoustic (PA) detection of melanoma CTCs in current research was based on energy transformation from absorbance in melanin nanoparticles into heat which generated acoustic waves. Evidence suggests that PA effects are more sensitive, have higher resolution, and can reach blood and lymph vessels at greater depths in vivo than current techniques. Additional research could enhance these techniques, including development of high-speed PAFC, with the possibility of use in clinics and at-home devices that could monitor CTCs in blood and lymph, potentially saving numerous lives. Furthermore, flow cytometry has the potential to provide new therapy methods by laser ablation of cancer cells.**

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## Introduction

Recently, there has been an increasing awareness of the need to produce a more efficient and sensitive method to detect early metastatic cells easily and quickly in vivo in cancer patients. Metastasis is one of the leading causes of death in cancer patients, and it is the primary cause of death in melanoma patients. However, conventional assays, like reverse transcriptase polymerase chain reaction (RT-PCR), can only detect possible metastasis after it has potentially spread to several parts of the body, and the disease is often incurable at that point (1). RT-PCR is also associated with a large number of false-positive signals from amplification of pseudo-genes and contamination, and is also not a direct method for detecting circulating tumor cells. Ex vivo circulating tumor cell (CTC) assays are also limited by a small blood sample volume that can miss approximately 5,000-50,000 cells because of low sensitivity (1). No current methods

exist that can assess relevant volumes of blood from clinically relevant sites like the primary tumor site; these problems are likely solvable by photothermal, photoacoustic, or fluorescent flow cytometry in vivo. Flow cytometry in vivo provides real-time assessment of cells in naturally flowing blood or lymph. It employs a laser and electronic detection device that can count cells as they pass through the laser beam. It is potentially a powerful research tool, because a method needs to be developed for earlier detection of CTCs that is sensitive enough to find merely one circulating tumor cell in blood or lymph so therapy could commence. Additionally, because cancer often reoccurs, even small amounts of CTCs need to be detected to ensure treatment is given.

Detecting circulating tumor cell counts in cancer patients is becoming an emphasized area of research dedication, because CTC counts are now considered a marker for metastasis development (1-3). Single-cell

detection is vital for early diagnosis of not only metastases, but also immune disorders and other diseases (2). In vitro, flow cytometry (FC) has revolutionized cell diagnostics, but without real-time evaluation and early detection of existing CTCs, and the with production of artifacts in vitro, like changes in cell-surface proteins, researches have hit a so-called glass ceiling of in vitro studies (2). Thus, researchers now seek to improve detection of CTCs by testing in vivo, potentially assessing the patient's entire blood volume with various flow cytometry methods (1). Previously, fluorescent labeling of CTCs in vivo was applied, but there has been much concern about unwanted immune responses and potential toxicity of the tags (1-3). Additionally, fluorescent labeling of the cells can only be used in superficial blood microvessels because of light scattering and an auto fluorescent background, but the superficial vessels have a slow flow rate (1, 2). Researchers believe a fast flow rate increases accuracy (1-3). Recent research indicates that nanoparticles--especially gold nanoparticles--could replace fluorescent markers and provide greater sensitivity without risking the toxicity of fluorescence (1-3).

Photoacoustic (PA) signals, a newly recognized detection method, are highly sensitive, and they greatly increase FC sensitivity while simultaneously reducing the threshold of labeling agent concentrations, which also reduces the risk of cytotoxicity (2). However, there has been an interest in developing a label-free method for photoacoustic flow cytometry (PAFC). Photoacoustic technology works because sound is generated by the absorption of light (2). Laser energy is absorbed by structures and transformed mostly into heat and then into amplified sound, so PA "listening" is potentially better than "seeing" the structures with fluorescence (2). PAFC technology shines a laser through a blood or lymph vessel and records the sound produced, which a marker of how much heat was absorbed. Additionally, PA technology is able to use melanin as an intrinsic marker because melanin is overexpressed in melanoma cells, and there is a PA signal contrast of melanin above the blood background (3). Photoacoustic in vivo flow cytometry can overcome previous limitations because it can detect weakly fluorescent naturally occurring molecules, like melanin, and is sensitive enough to detect a single cell. Several other studies have demonstrated that PAFC techniques are safe and significant (3). An ultimate goal is to provide this technology widely available, because the signals can be detected with a standard ultrasound machine. In vivo limitations of PAFC include discrimination of melanin signals in melanoma and background signals from blood and other noise.

### Recent Progress

Researchers at the University of Arkansas for Medical Sciences have developed a method for non-toxic, in vivo, label-free detection and eradication (1) of CTCs in metastatic melanoma cells using photoacoustic imaging (1-3). Photoacoustic imaging provides higher spatial resolution and can be used in deeper tissue because it utilizes endogenous chromophores—like hemoglobin and melanin—which have selective light absorption, and exogenous nanoparticles that provide contrast of signals (1). These researchers have developed photoacoustic flow cytometry (PAFC) that potentially detects circulating squamous cell carcinoma cells in real-time in vivo by labeling them with gold nanorods (GNR) in slow lymph flow (2), and can also detect unlabeled melanoma cells (1-3). They also changed from a low laser pulse rate to a high pulse-repetition-rate diode laser (1-3) which increased sensitivity to melanoma CTC count in blood circulation and could be integrated with laser ablation (1). Fast flow is crucial here because it reduces the rate of false-negative PA signals and allows multiple signals from the same cell, which increases detection sensitivity through noise reduction (3). Although most of the research was done in blood (1-3), some research focused on lymph flow (2). Additionally, researchers used photothermal (PT) thermolens signals (1-3) to indicate average melanin content in tumor cells, and PT image structures to indicate the melanin spatial distribution in individual cells (1). PT technology was developed by other researchers and was not the primary focus of this research; it was used to visualize melanin nanoparticles. In vivo detection of a melanoma cell injected with an i.v. absorbance versus red blood cell (RBC) absorbance was possible because RBCs produced a photoacoustic signal with greater amplitude at a wavelength of 865 nm, and melanoma cells produced photoacoustic signals with a significantly higher wavelength at 639 nm (1). Additional research improved the speed of PAFC using a high-pulse repetition fiber laser operating at a higher wavelength of 1064 nm (3).

Results from studies disagreed about how sensitive PAFC detection of cells was. One study said PAFC can detect about 82% of melanoma cells without labeling in the blood ( $12 \pm 1.3$  cells/minute) and slightly more ( $14.6 \pm 1.4$  cells/minute) with gold nanorod (GNR) labeling (1), while another paper suggests that label-free PAFC can miss up to 50-60% of cells (3). Studies were also inconsistent regarding mice with high versus low skin pigmentation; one study showed decreased signal rate for high-pigmentation cells, at about 10 cells/minute (1), while another said that PAFC missed most low-pigmentation cells (3). The latter group suggested nanoparticle tagging and subsequently bringing a magnet close to the vein would increase PA signals. They proposed this method worked because targeting the CTCs with nanoparticles causes the cells to aggregate, and the

labeled cells were then slowed or trapped in the presence of a strong magnetic field, which increased PA signal contrast approximately ten-fold (3). PAFC was also used to detect and damage metastatic cells during tumor progression *in vivo*, and melanoma CTCs were counted in an ear blood vessel and skin blood vessels, both of which had tumors near them (1). Results showed that CTCs were detected weeks before any signs of metastasis showed up in tissue samples (1). Regarding irradiation, daily laser treatments were given in the abdominal skin blood vessels for two hours, and after four weeks no evidence of metastasis was found in mice with skin tumors (1). Irradiation included capturing CTCs and their clusters by gentle mechanical squeezing of the mouse blood vessel.

Other research has focused primarily on FC in lymphatics because the lymphatic system is crucial in the spread of metastatic cells like melanoma. Researchers demonstrated *in vivo* that the lymphatic route shows the earliest stage of metastasis disease, even before metastasis can be detected in the sentinel lymph nodes, and that the lymph system spreads diseased cells to distant organs in the late stage of metastasis (2). However, assessing cells in lymphatics is not a widely-understood area of FC. FC *in vivo* in the lymph system is also limited because of natural light scattering, fluctuations in cell velocity and position, a slower flow rate than in blood, and oscillating flow between systole and diastole. Researchers introduce cell counting in lymph vessels with natural cell-focusing and PA effects detectable with an ultrasound technique. PAFC in the lymph system was developed because they discovered that cells can be focused naturally and hydrodynamically by irradiation near a valve nozzle if they form a single-file flow. They integrated PAFC with time-division multiplexing (TDM), photothermal (PT) techniques, and fluorescence, so they could analyze cells with different absorption, scattering properties, and fluorescence. PA visualization of lymph at 639 nm was optimal, because blood absorption is maximal at 580 nm. This discovery may provide a way to determine lymph vessel's margins in deep locations. Additionally, strong focusing of the laser beam generated large PA signals. The determination of melanoma cells was based on laser-induced PT and resulting PA signals using melanin as the intrinsic marker. PT signals from cytochromes, hemoglobin, and melanin are signature images that can be compared to known signals. PA maps detected single micrometastases in lymph nodes *in vivo* during the second week after inoculation, which has huge significance for early diagnosis of metastasis. They found that melanoma cells move often as small aggregates. Furthermore, they found that endogenous cellular absorption can be used as intrinsic cell-specific markers that have unique "signatures" and produce enough sound to detect with conventional ultrasound techniques. They

discovered that the sensitivity of PAFC in single-cells is at least four orders of magnitude better than absorbance spectroscopy for the same condition (2).

### Discussion

This technology should be readily translation into use for humans with the possibility of using a portable device that could attached to a blood or lymph vessels and detect metastasis, assess therapy, and provide cell diagnostics (2, 3). Research suggests a fiber laser at 1064 nm with 0.5 MHz repetition rates are a promising source of portable clinical PAFC devices, with usages including earlier diagnosis of melanoma CTCs, monitoring the primary tumor, detection of reoccurrence, detection of residual disease, and monitoring therapy effectiveness before, during, and after therapy (3). Although the PACF technique may not detect all CTCs as early as desirable yet, it still has a large clinical relevance because it detects CTCs sooner than currently used methods, and can be used in blood testing for early melanoma diagnosis and reoccurrence diagnosis. Using PAFC cell focusing phenomenon and PA effects in single cells has the potential to address metastasis and many other related issues, in particular, lymph testing *in vivo* (2), and PA has still demonstrated higher resolution, depth, and sensitivity than current assays (3). An estimated 70-100 CTCs were detected merely four days after inoculation in several mice, providing evidence that that PAFC is an effective method for detecting potential metastatic cells in blood and lymph flow very early after their release into circulation (3). However, the sensitivity threshold is dependent on flow rate and monitoring time; one CTC in whole mouse blood volume took three hours (3). Eventually, high-speed PAFC should be developed to reduce time required to monitor the whole blood volume even in deep vessels, and should have enough sensitivity to detect even a few CTCs per whole blood volume (3).

The most notable aspect of this research is the detection of real-time label-free metastatic melanoma cells *in vivo* (2). The detection rate is approximately equal to detecting one melanoma cell in the background of  $10^6$  WBCs, which is unprecedented sensitivity using any existing technique (2). In large lymph vessels, further research should be able to improve the sensitivity threshold *in vivo* that far exceeds existing conventional assays *ex vivo*. PACF specificity can be enhanced by the use of multicolor nanoparticles with different absorption spectra, especially gold-based probes, because gold is nontoxic and does not photobleach or blink. Future research could develop PACF even beyond diagnosis to include *in vivo* lymph purging by elimination with a laser of cancer cells labeled with nanoparticles (2). It should also realize spectral fingerprints with label-free identification of cancer cells. Further improvements

could be made in the signal-to-noise ratio of melanin signals over blood background by enhancing melanin synthesis using drugs, enhancing melanin activators, capturing large CTCs, improving laser pulse width to match the acoustic and thermal relaxation times of melanin nanoparticles, and increasing laser-induced nanoparticle clustering (1). Future research could focus more on clinical trials, including blood screening for CTCs and cancer reoccurrence, and detection of cancer cells around surgical sights to evaluate residual cancer cells (1, 3). It is not clear whether laser ablation is effective enough to be used alone, and for now it should be used in conjunction with chemotherapy and radiation; however, future studies may enhance these techniques enough to use lasers to prevent metastasis and damage CTCs. The ultimate goal is to be able to bring this technology to the bedside.

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