

Detection of Melanoma Cells In Vitro Using an Optical Detector of Photoacoustic Waves

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Background and Objective: Circulating tumor cells have been shown to correlate positively with metastatic disease state in patients with advanced cancer. We have demonstrated the ability to detect melanoma cells in a flow system by generating and detecting photoacoustic waves in melanoma cells. This method is similar to flow cytometry, although using photoacoustics rather than fluorescence. Previously, we used piezoelectric films as our acoustic sensors. However, such films have indicated false-positive signals due to unwanted direct interactions between photons from the high laser fluence in the flow system and the film itself. We have adapted an optical detection scheme that obviates the need for piezoelectric films.

Study Design/Materials and Methods: Our photoacoustic system comprised a tunable laser system with an output of 410–710 nm with a pulse duration of 5 nanoseconds. The light was delivered by optical fiber to a glass microcuvette that contained saline buffer suspensions of melanoma and white blood cells. We used a continuous HeNe laser to provide a probe beam that reflected off of a glass and water interface in close proximity to the microcuvette. The beam was detected by a high-speed photodiode. When a photoacoustic wave was generated in the microcuvette, the wave propagated and changed the reflectance of the beam due to index of refraction change in the water. This perturbation was used to detect the presence of melanoma cells.

Results: We determined a detection threshold of about one individual melanoma cell with no pyroelectric noise indicated in the signals.

Conclusions: The optical reflectance method provides sensitivity to detect small numbers of melanoma cells without created false-positive signals from pyroelectric interference, showing promise as a means to perform tests for circulating melanoma cells in blood samples. *Lasers Surg. Med.* 42:274–281, 2010. © 2010 Wiley-Liss, Inc.

Key words: cancer; metastasis; optical reflectance; photoacoustic; Q-switched

INTRODUCTION

Circulating tumor cells (CTCs) are those cells that detach from a primary or secondary tumor and spread to distant organs via the blood or lymph systems [1–5]. CTCs have been shown to correlate with metastatic disease state and

their detection and quantification may be used by clinicians to optimize the therapy of cancer patients. CTCs may occur as one cell among millions of normal blood cells and may originate from solid tumors or hematological malignancies such as leukemia. Many methods have been proposed to find CTCs, but due to their rare occurrence no accepted method has been implemented in clinical practice.

RT-PCR, immunomagnetic separation, micro-fluidics, and automated digital microscopy have all been investigated as means for CTC detection [6–8]. All of these methods suffer from a combination of sensitivity problem, the need for specialists for sample analysis, and slow processing time. RT-PCR in particular has been hampered by high cost and difficult preparation. While it has successfully been used to detect CTCs in a research setting, its peculiar absence in clinical diagnosis is due to these short comings.

Automated digital microscopy has been used along with fiber-optic array scanning, although the need for single cell passes requires several hours for testing a single sample. Immunomagnetic separation has been used widely in research and has even manifested as a commercial device, the CellSearch system. However, this system requires manual imaging of suspect cells and takes hours to process a single sample.

Flow cytometry, however, is used routinely to count leukemia cells using fluorescence detection [9]. This method uses high flow rates of single cells through a detection volume within which cells pass. This method is effective for finding large numbers of CTCs, although rare CTCs from solid tumors would probably pass unnoticed. For leukemia, CTCs are not rare events, as the disease itself is characterized by a proliferation of malignant cells in the blood stream.

We have developed a system for detecting circulating melanoma cells (CMCs) exploiting the natural chromophore,

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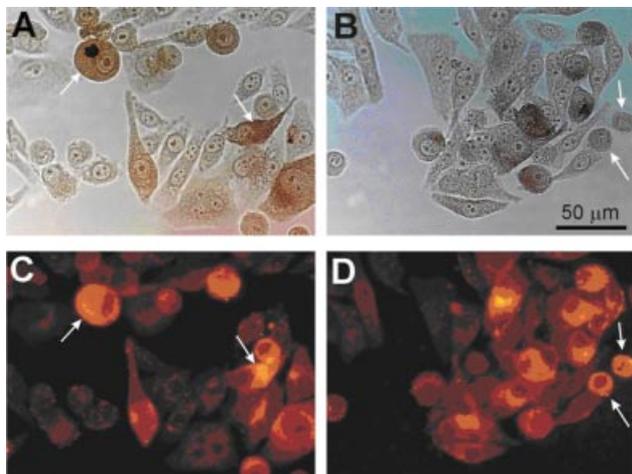


Fig. 1. Pictures of human malignant melanoma cell line HS 936.T. **A,B:** Transmitted light pictures of two different cell cultures. **C,D:** Immunofluorescence of the same cells stained for tyrosinase. Note that the darker cells under transmitter light (A and B) do not represent the cells that express the most tyrosinase (arrows in AC vs. BD). [Figure can be viewed in color online via www.interscience.wiley.com.]

melanin, that is present in most melanoma cells (Fig. 1) [10]. It has been estimated that over 95% of all melanomas are pigmented, enabling the photoacoustic process to work [11,12]. Figure 1 shows the melanoma cell line we used in these experiments. Figure 1A,B shows melanin pigment directly, while immunofluorescence stains in Figure 1C,D show the presence of tyrosinase, a melanin precursor.

In our method for CMC detection, we circulate a suspension of cells in a closed loop comprised of a peristaltic pump, silicone tubing, and a glass flow chamber. This glass flow chamber is irradiated with nanosecond-pulsed laser light, generating photoacoustic waves in suspended pigmented cells. Typically, this cell suspension would be the buffy coat separated from whole blood of cancer patients. The buffy coat is composed of white blood cells that have no inherent optical absorption. If the whole blood sample contained CMCs, they would separate with the buffy coat. Separation of the buffy coat is a standard practice and has been used in CTC research [13]. Due to density differences in blood components, centrifugation separates red blood cells, white blood cells, plasma, and lipids. CTCs have a similar density to white blood cells and therefore separate within the buffy coat. We used a Histopaque solution with a density of 1.077 g/ml to further demark the red blood cells from the buffy coat, allowing us to remove the buffy coat with a micropipette. We estimate from current work that we can recover 80–90% of melanoma cells spiked in whole blood after centrifugation.

Thus, in the absence of pathological conditions that cause optical absorption in leukocytes, photoacoustic events generated in this flow system indicate the presence of melanoma cells in the circulatory system. Since the white blood cells have no absorption, we can pass thousands of cells through the active sensing area without compromising

the sensitivity of pigmented cell detection. Thus, we have been able to test 10-ml samples of blood within a few minutes.

In metastatic patients, the number of CMCs is variable. As long as the suspension is dilute enough so that only one cell is in the laser beam path at any given time, the number of CMCs can be determined by counting the acoustic events generated in the flow system. If there is a continuous stream of such cells, the suspension can be made more dilute so that single cells can be counted. A continuous stream is obvious by the steady-state photoacoustic signal it generates. Such steady-state signals have been seen in our laboratory tests on high-concentration melanoma cells.

One difficulty in our detection system arose from using piezoelectric films in our flow chamber. The original design of our system used polyvinylidene difluoride (PVDF) film as a piezoelectric element. While PVDF is a sensitive detector of transient pressure waves, it was subject to a pyroelectric effect that generated voltages due to photon scatter onto the film. In addition, slight misalignment of the system caused burning of the film due to high laser fluence. These burns induced blackened regions on the film that induced their own photoacoustic events, increasing the false-positive signals.

In order to overcome these difficulties, we built an optical sensor for photoacoustic detection. We used the method developed by Paltauf et al. [14], in which a continuous reference beam reflected off of a glass and water interface. This reflection was set near the critical angle so that a slight perturbation in the index of refraction of water would cause a large change in reflectance. We launched such a reference beam into a prism on which our flow chamber rested. Thus, photoacoustic events induced in melanoma cells caused transient changes in optical index of refraction, which in turn caused a transient change in reference beam reflectance that was sensed by a fast photodiode. We tested this system on black latex microspheres for calibration and on cultured melanoma cells suspended in saline buffer and in a white blood cells suspension. We found that this system had a threshold of a few melanoma cells, supporting its use as a means to detect CMCs in blood samples.

MATERIALS AND METHODS

We designed a photoacoustic system for CMC detection using the aforementioned optical detector of acoustic waves and a tunable laser system. While the eventual clinical system will incorporate a flow system, we restricted our work to static conditions to evaluate the ability of the optical detector to sense small concentrations of melanoma cells.

Experimental Apparatus

The experimental apparatus for the photoacoustic detection of biological microparticles is shown in Figure 2. A frequency tripled Nd:YAG laser pumping an optical parametric oscillator (OPO) (Vibrant 355 II, Opotek, Carlsbad, CA) was employed to provide 532-nm laser light with a pulse duration of 5 nanoseconds. Beam energy entering the flow cell was about 7 mJ with a pulse repetition

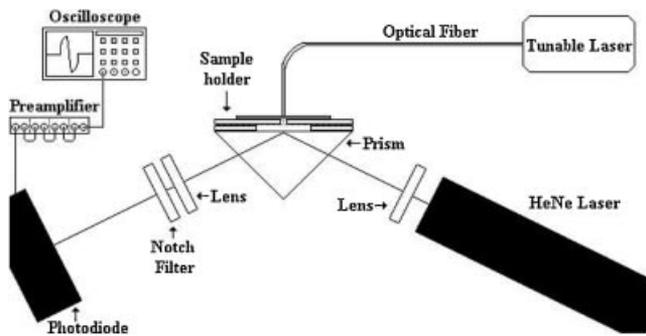


Fig. 2. Schematic representation of photoacoustic detection set-up.

rate of 10 Hz. The pulsed light beam was delivered via a 1,000- μm optical fiber. Laser fluence in the flow chamber was 0.4 J/cm^2 with a spot size of 1.5 mm. A glass sample holder with an inner diameter of 1.2 and 1 mm depth was made by grinding a microscope slide and was used to contain black latex microspheres or melanoma cells (Fig. 2). Acoustic pressure waves were detected by an optical stress transducer.

The design of the optical stress transducer used in our experiments is shown in Figure 3. It is based on the design of Paltauf et al. and is completely described in Ref. [14]. The method relies on the probe laser beam being placed near the critical angle of total internal reflection determined by the indexes of refraction of glass and water, the constituents of the interface. Perturbations in the index of refraction of water induced by wave action from photoacoustic pressure causes changes in probe beam reflection sensed by a photodiode.

The HeNe laser beam was reflected at the surface of a right angle glass prism. A lens with a focal length of 120 mm was used to focus the beam to an elliptical spot on the glass–water interface. A second lens imaged the HeNe laser spot on the prism surface onto the active area of the 1-GHz photodiode (1601-FS-AC; New Focus, San Jose, CA). Photoacoustic waves arrived at the detector after passing the sample in transmission mode. The photoacoustic signals from the optical stress transducer were displayed by a 200-MHz oscilloscope (TDS 2034B; Tektronix, Wilsonville, OR) triggered by a photodiode (DET10A; Thorlabs, Newton, NJ) with a 1-nanoseconds rise time. The signals were amplified with a gain of 125 via 350 MHz amplifier (SR445A; Stanford Research Systems, Sunnyvale, CA).

The microcuvette was a cylindrical hole ground in the center of the microscope slide with a diameter of 1.2 mm (Fig. 3). The bottom and top of the microcuvette were sealed with thin vinylidene chloride film (Saran Wrap; Dow Chemical Corp, Midland, MI) with a thickness of $16 \mu\text{m}$ and attached with silicone sealant and a glass cover slip with a thickness of $150 \mu\text{m}$ attached with vacuum grease. The microcuvette was separated from the prism surface by a thin layer of water, approximately 1 mm thick. This chamber had a volume of about 10 ml. However, the volume of laser irradiation was about 1 ml. This volume was determined using simple geometric analysis of the following parameters: fiber numerical aperture—0.37, radius of optical fiber—1,000 μm , and height of fiber above microcuvette—1,000 μm .

In order to assess the sensitivity of the photoacoustic device, we used black latex microspheres due to their similarity to melanoma cells and due to the fact that they are a repeatable standard available commercially. We

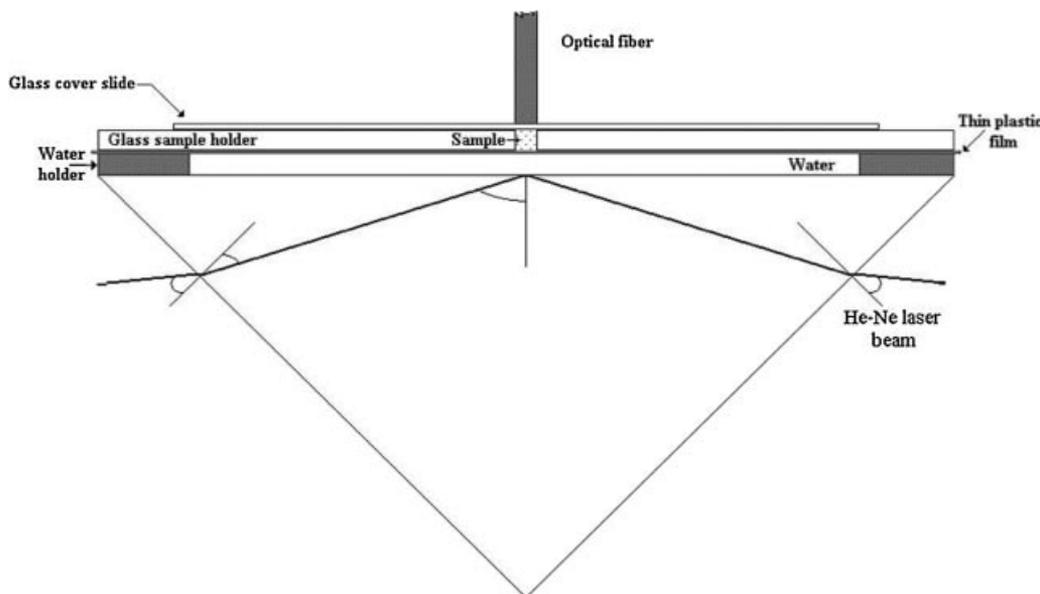


Fig. 3. Photoacoustic cell including irradiated microparticles and prism where the reflection of Fresnel occurs.

irradiated samples of 5, 20, 50, and 100 microspheres/ml suspended in Tyrode's buffer. We also irradiated pure buffer in order to assess whether it had any intrinsic absorption that would interfere with the photoacoustic testing. In previous work, we verified that white microspheres gave no photoacoustic signal.

Photoacoustic Targets

Black polystyrene latex microspheres (2-BK-7000; Interfacial Dynamics Corp.) with a diameter of 10 μm were used to calibrate the photoacoustic detection system. Microspheres were suspended in 10 ml of Tyrode's buffer (125 mM NaCl, 4.7 mM KCl, 1.4 mM CaCl₂, 20 mM NaHCO₃, 0.4 mM NaH₂PO₄, 1.0 mM MgCl₂, 10 mM D-glucose, pH 7.4) resulting in concentrations ranging from 5 to 200 microspheres/ml.

Human malignant melanoma cells, HS 936 (American Type Culture Collection, Manassas, VA), were cultured in suspension with RPMI-1640 growth medium (Sigma, St. Louis, MO) + 10% fetal bovine serum (BSA), incubated at 37°C, and were suspended in 10 ml of Tyrode's buffer. The diameter of the melanoma cells was determined by transmitted light microscopy and immunostaining and was about 15 μm . The immunostaining process was as follows: melanoma cells were cultured for 3 days on a thin 25-mm microscope cover glass (Fishebrand; Fisher Scientific) fixed with 3.7% formaldehyde (Sigma) for 10 minutes, washed five times for 5 minutes with phosphate-buffered saline (PBS). Immunostaining was performed by blocking the sections with 2% BSA in PBS and incubating with the appropriate primary antibodies (Tyrosinase-ab738; Abcam, Cambridge, MA), at 4°C for 24 hours, followed by incubation with fluorochrome-conjugated secondary antibodies (Zymed, San Francisco, CA) for 2 hours at room temperature, approximately 23°C, washing in PBS, and finally laying the cover slips on the slides with Prolog Antifade (Molecular Probes, Eugene, OR). Images were digitally photographed at 40 times magnification with an upright BX51WI Olympus microscope (Olympus, Tokyo, Japan) equipped with an Axiocam MRC. Figure 1 shows the human malignant melanoma cell line HS 936.T. Figure 1A,B shows transmitted light photos of two different cell cultures, and Figure 1C,D shows immunofluorescence images of the same cells stained for tyrosinase. Note that even when the cells are not dark under the transmitted light, they express more tyrosinase indicated by arrows in Figure 1A–C versus Figure 1B–D.

Signal-to-noise ratio (SNR) was determined by the peak-to-peak amplitude of the photoacoustic wave and the noise. Noise level was determined by the mean-squared error of approximately 1,000 data points obtained from irradiating Tyrode buffer.

Detection Threshold of Cultured Melanoma Cells

We performed tests on cultured melanoma cells to determine the detection threshold of the photoacoustic device. These cells were suspended in Tyrode's buffer. We irradiated concentrations of cells including 10, 20, and 50 cells/ μl . Additionally, we suspended two melanoma cells among approximately 1 million white blood cells in 1 μl of

buffer, as this corresponds to the clinical testing paradigm noted above.

RESULTS

Calibration of Photoacoustic Device Using Latex Microspheres

The latex microsphere suspensions were introduced into the photoacoustic system to characterize the sensitivity of the device. In Figure 4a–e, the waveforms for buffer solution and five concentrations of microspheres are shown. Experiments were repeated five times and error bars indicate the standard deviation. The calibration curve, Figure 4f, indicates a detection threshold of about 1 microsphere, where 1 microsphere gives a photoacoustic waveform with an SNR of about 5:1.

Detection Threshold of Cultured Melanoma Cells

In Figure 5a–c, the continuous black line corresponds to 10, 20, and 50 melanoma cells/ml and the continuous red line corresponds to Tyrode's solution. The waveform of Tyrode's solution is flat and constituted the noise floor. The trials resulted in a detection threshold of fewer than 10 cells/ml of Tyrode's buffer. Taking into account the total irradiated volume within the photoacoustic chamber, it can be estimated that only a few melanoma cells are necessary to maintain a strong signal during a particular data-acquisition period. An SNR is used to display the peak voltage to eliminate increased signal strength due to noise as shown in Figure 5f.

Figure 6 shows a digital micrograph of the detection cuvette within which lies two cultured melanoma cells. The cells were suspended among a million white blood cells. The accompanying graph shows a robust photoacoustic signal with an SNR of about 3:1 arising from the melanoma cells, as shown by the black trace, while the white blood cells show no signal with a noise floor of about 3 mV.

DISCUSSION

CTCs have been studied recently as indicators of metastatic disease [15,16]. Detection of CTCs poses a difficult problem as their incidence, even in late-stage cancer patients, is small and their numbers are often limited to a few in a 10-ml blood sample. Furthermore, the difficulty in detecting CTCs has resulted in a lack of understanding of their etiology. While there is evidence that the numbers correlate to metastatic disease state, this correlation is not precisely known. Open questions about CTC incidence include whether they are constantly present in the blood stream and whether they originate in the blood or lymph systems. Having a sensitive and specific method to detect CTCs would not only provide valuable information for clinical management of metastatic cancer, but also provide a tool for studying cancer biology and the progression of advanced cancer.

Microsphere Calibration

The microsphere calibration was performed to show system sensitivity in the most repeatable manner, with

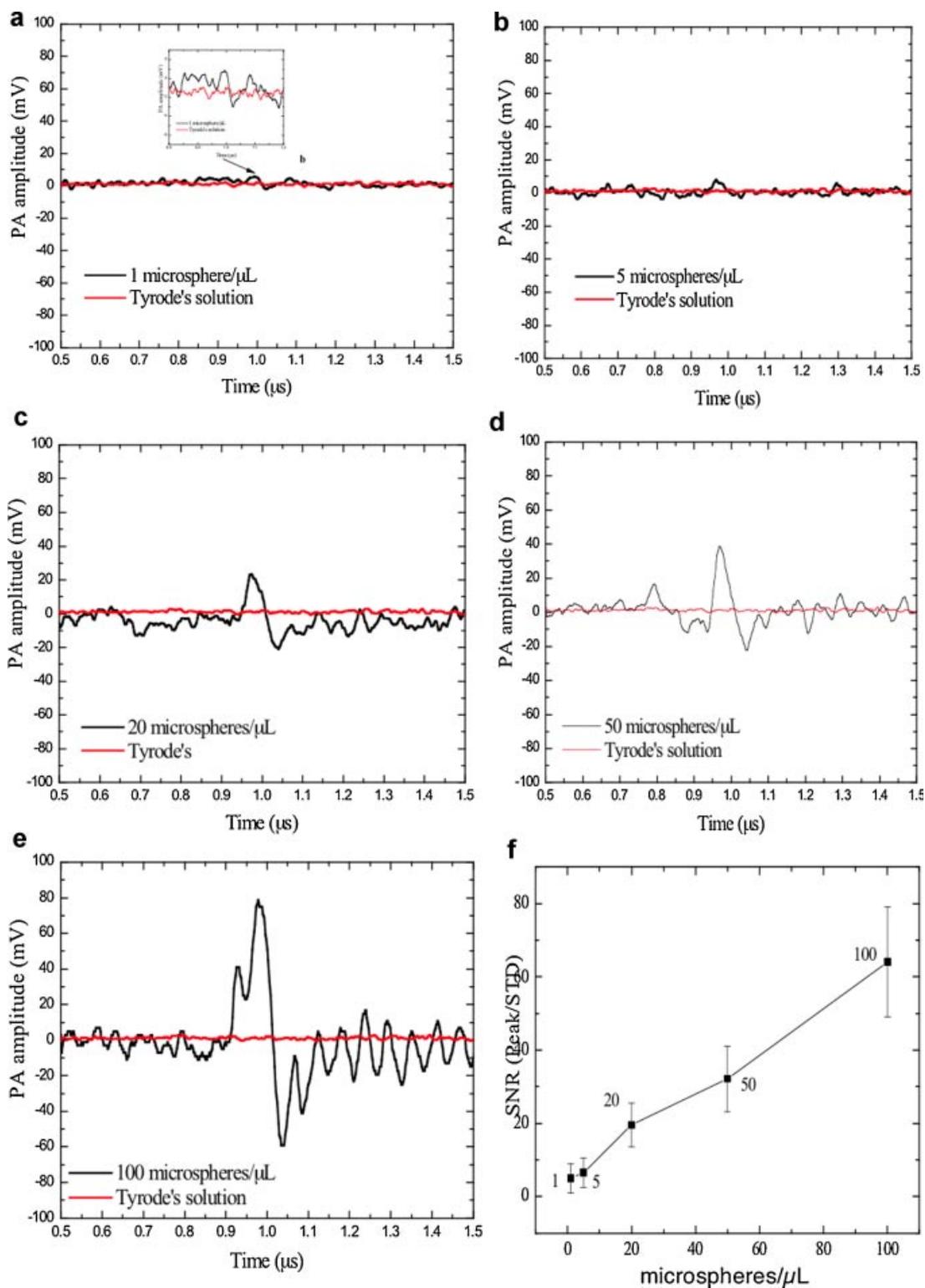


Fig. 4. **a–e**: Photoacoustic waveforms for five concentrations of latex microsphere suspensions. **f**: The SNR versus serial dilutions of microsphere suspension shows a detection threshold of a single microsphere. [Figure can be viewed in color online via www.interscience.wiley.com.]

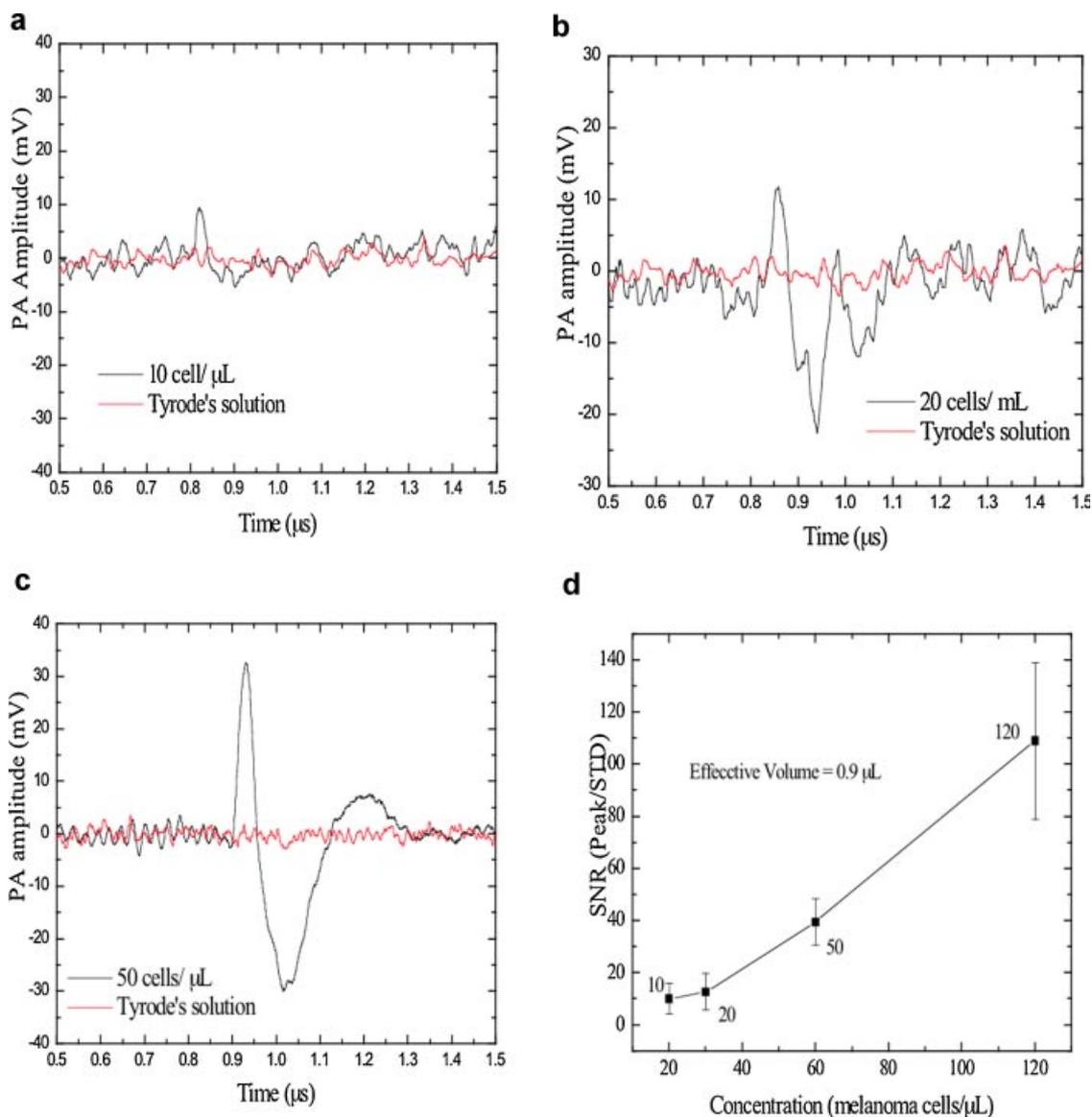


Fig. 5. **a–c:** PA waveforms for the Tyrode's buffer and lowest concentrations of cultured human melanoma cells. **d:** Peak amplitude of the PA waveform for melanoma cell suspensions of various concentrations indicating an SNR of about 10:1 for 10 melanoma cells. [Figure can be viewed in color online via www.interscience.wiley.com.]

the assumption that microspheres had little variation in dye content. This assumption is probably valid within microsphere batches. All experiments performed in this article were performed on the same batch for this reason. Figure 4 shows the decreasing signals due to dilution. The single sphere shows a weak signal that normally would not have been considered above threshold, although previous work had trained the experimenters to recognize these signals. In any case, microsphere detection threshold is not important, since there is no practical way to correlate microsphere absorption with CMCs. In fact, it is unclear whether the dye is uniform throughout the sphere or restricted

to the surface. Thus, it is not possible to determine absorption coefficient of individual spheres. In fact, bulk absorption is not a simple matter, as the relationship of scattering and absorption is unknown. However, the calibration work served to show general system response to suspended microparticles in a repeatable fashion, in anticipation of performing tests on melanoma cells.

Melanoma Cell Detection

Figures 5 and 6 show a set of photoacoustic signals induced in suspended melanoma cells. The SNR indicates that it may be possible to detect single melanoma

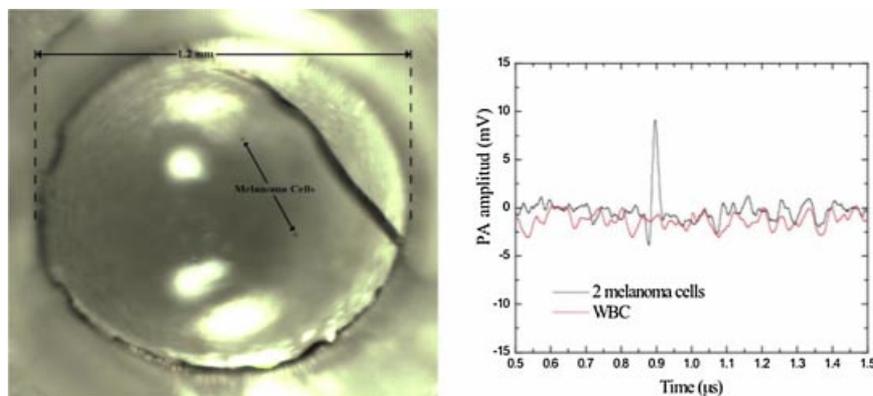


Fig. 6. The micrograph at left shows the PA cell containing two cultured melanoma cells. The graph at right shows the relatively at line of white blood cells (WBCs) in red, while the melanoma cells in suspension show a 10-mV signal indicated by the black trace. [Figure can be viewed in color online via www.interscience.wiley.com.]

cells, providing that there is sufficient pigmentation within the cells.

Biological Variability of Melanin Content

Our photoacoustic method for the detection of CMCs is simple, rapid, and detects the melanoma cell itself, rather than a biological or chemical marker related to the cancer, such as prostate-specific antigen for prostate cancer screening. There are, however, limitations to this technique. First, this label-free method is limited to melanoma, since it is the only cancer type that has an intrinsic optical absorber. Even within the study of melanoma, there is great variation in the amount of melanin within melanoma cells. Some melanomas originate as amelanotic cells, while others lose their pigmentation over time. We have been working with researchers to extend this photoacoustic method to non-pigmented cancers by attaching nanoparticles to cancer cell surface antigens, thus providing an extrinsic absorption that is well characterized. If such non-melanoma cancers can be targeted, the same technique can be extended to other cancer types as well as enhancing the photoacoustic signal from amelanotic melanoma.

Statistical Classification of CMCs

A second shortcoming is that this method is not imaging. While this aspect greatly simplifies the implementation, it brings up a question of specificity of the test. Since individual cells are not inspected, as in other methods such as immunomagnetic separation, we have developed a statistical classifier based on the distinct absorption spectra of possible chromophores. As leukocytes, and most cells in general, have no significant chromophores with regard to the optical absorption of melanin, the only other possible significant absorption can arise from individual red blood cells present in the buffy coat as a result of incomplete centrifuge separation. However, hemoglobin, whether oxygenated or deoxygenated, has distinct absorption from melanin. Using two laser wavelengths, such as 422 and 530 nm, we can monitor the photoacoustic response

at these two wavelengths and classify the absorbing particle according to the ratio of amplitudes. For instance, a red blood cell will have much larger absorption at 422 than 530 nm, on the order of 11:1 while melanin has a ratio of $<2:1$. As these laser wavelengths are the isosbestic points of oxygenated and deoxygenated hemoglobin, this classification scheme is independent of the oxygenation state of the blood cell. Additionally, the wavelength 532 nm can be used since its absorption is close to that at 530 nm, with the practical benefit that it is the second harmonic of an Nd:YAG laser and is thus readily available. We have tested a classical multivariate classification scheme on whole and thermally coagulated blood [17] and have extended this effort to a Bayesian classifier on pigmented and vascular lesions *in vivo* [18]. We are building up a histogram of individual melanoma and red blood cells in order to implement the classifier for CMCs. We anticipate the mean of the two distributions of cell types to be separated by 5–7 standard deviations, thus making this a robust classifier using classical or Bayesian methods.

Quantification of CMCs

The experiments in this article were meant to determine the feasibility of the optical stress transducer as the acoustic sensor for the detection of CMCs. While PVDF is sensitive, high laser fluence can easily interact with the film by minor misalignment of the device or by scattering induced by white blood cells. Thus, we used the optical stress transducer to alleviate these problems. Our task was to determine if this transducer had the ability to detect single melanoma cells in order to count them in a flow system. As fluid flow introduces variability to the measurement, for this evaluation we chose to test the set-up on static samples. These samples were placed in microcuvettes we made by grinding small holes in glass microscope slides. All tests were performed within minutes of placing the suspensions in order to maintain a more even distribution in the fluid. We found that after a period of time, on the order of an hour, the microspheres would settle at the bottom of the microcuvette.

With the current set-up, we cannot quantify melanoma cells accurately, not only due to the variability of melanin content in CMCs, but also due to the small changes in sensitivity of the device that depends on precise alignment, particularly of the probe HeNe beam being set near the critical angle of total internal reflection. While care is taken to set the beam angle near this angle, our precision is on the order of a few tenths of a degree, resulting in deviations of sensitivity from experiment to experiment. The method we will employ to quantify CMCs will be to count them under flow by correlating transient photoacoustic events with CMC number. CMCs are generally rare in blood samples, often fewer than 10/ml. With an active area of about 10 ml, there can be as many as 100 CMCs/ml before the transient events combine into a fairly steady-state signal. In the case of more than 100 CMCs/ml, a steady-state signal will signify the need to dilute the cell suspension until transient photoacoustic events are detected. CMC concentration can then be deduced by the number of events and the dilution of the suspension. Analysis of the amplitude will never be feasible since error introduced by biological variability of melanin and variability of system response will preclude quantification. Thus, we seek to count individual melanoma cells in flow passing through the photoacoustic laser beam.

The current studies show that the optical stress transducer, while immune to the problems of piezoelectric films, indicates a detection threshold at or near single melanoma cells. This sensitivity is insufficient, as we need robust detection of single-cell events, taking into account the variability in melanin content and slight changes in alignment. Thus, we seek to improve sensitivity by a factor of 10 in order to detect single melanoma cells with confidence. We may increase amplification of the photoacoustic signal. While this may sacrifice linearity, this process looks for the presence or absence of a photoacoustic event, thus absolute measurement of amplitude is not important. The limitation of amplification, however, is determined by the ability to decrease random noise by signal averaging. Currently, the melanoma cell is in the beam path for about 2 seconds. With a pulse repetition rate of 10 Hz, we can only average fewer than 20 pulses. However, we may be able to improve photoacoustic signal by attaching gold nanoparticles to melanoma cells after the centrifugation procedure. Preliminary studies show this method increases photoacoustic amplitude and thus sensitivity by a factor of 3–5.

While these studies do not prove that the optical stress transducer should replace piezoelectric film as the sensor for CMC detection, the technique shows promise and will be evaluated further using the aforementioned ideas.

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REFERENCES

- Gilbey A, Burnett D, Coleman R, Holen I. The detection of circulating breast cancer cells in blood. *J Clin Pathol* 2004; 57:903–911.
- Woele U, Breit E, Zafrakas K, Otte M, Schubert F, Miller V, Izbicki J, Lning T, Pantel K. Bi-specific immunomagnetic enrichment of micrometastatic tumour cell clusters from bone marrow of cancer patients. *J Immunol Methods* 2005; 300:136–145.
- Fisher B, Redmond C, Fisher E, Bauer M, Wolmark N, Wickerham D, Deutsch M, Montague E, Margolese R, Foster R. Ten year results of a randomized clinical trial comparing radical mastectomy and total mastectomy with or without radiation. *N Eng J Med* 1985;312:674–681.
- Krivacic R, Ladanyi A, Curry D, Hsieh H, Kuhn P, Bergsund D, JF K, Barbera T, Ho M, Chen L, Lerner R, Bruce R. A rare-cell detector for cancer. *Proc Natl Acad Sci USA* 2004;101: 10501–10504.
- Gusterson B, Ott R. Occult axillary lymph-node micrometastases in breast cancer. *Lancet* 1990;336:434–435.
- Koyanagi K, O'Day S, Gonzalez R, Lewis K, Robinson W, Amatruda T, Wang H, Elashoff R, Takeuchi H, Umetani N, Hoon D. Serial monitoring of circulating melanoma cells during neoadjuvant biochemotherapy for stage iii melanoma: Outcome prediction in a multicenter trial. *J Clin Oncol* 2005;23:8057–8064.
- Arenberger P, Arenbergerova M, Vohradnikova O, Kremen J. Early detection of melanoma progression by quantitative real-time rt-pcr analysis for multiple melanoma markers. *Keio J Med* 2007;57:57–64.
- Hsieh H, Marrinucci D, Bethel K, Curry D, Humphrey M, Krivacic R, Kroener J, Kroener L, Ladanyi A, Lazarus N, Kuhn P, Bruce R, Nieva J. High speed detection of circulating tumor cells. *Biosens Bioelectron* 2006;21:193–1899.
- Givan AL. *Flow Cytometry: First Principles*. Wiley Liss: 2001.
- Weight R, Dale P, Caldwell C, Lisle A, Viator J. Photoacoustic detection of metastatic melanoma cells in the human circulatory system. *Opt Lett* 2006;2998–3000.
- Jacques S, McAuliffe D. The melanosome: Threshold temperature for explosive vaporization and internal absorption coefficient during pulsed laser irradiation. *Photochem Photobiol* 1991;769–775.
- Wain E, Stefanato C, Barlow R. A clinicopathological surprise: Amelanotic malignant melanoma. *Clin Exp Dermatol* 2008;33:365–366. [9] Vitkin I, Woolsey J, Wilson B, Anderson R. Optical and thermal characterization of natural (*sepia officinalis*) melanin. *Photochem Photobiol* 1994; 455–462.
- Lara O, Tong X, Zborowski M, Chalmers JJ. Enrichment of rare cancer cells through depletion of normal cells using density and flow-through, immunomagnetic cell separation. *Experim Hematol* 2004;891–904.
- Paltauf G, Schmidt-Kloiber H, Guss H. Light distribution measurements in absorbing materials by optical detection of laser-induced stress waves. *Appl Phys Lett* 1996;69:1526–1528.
- Cristofanilli M, et al.. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *New Engl J Med* 2004;781–791.
- Check W. Circulating tumor cell tests—boon or bust? *Cap Today* 2005;1:76–86.
- Talbert RJ, Holan SH, Viator JA. Photoacoustic discrimination of viable and thermally coagulated blood using a two wavelength method for burn injury monitoring. *Phys Med Biol* 2007;52:1815–1829.
- Swearingen J, Holan SH, Feldman M, Viator JA. Photoacoustic discrimination of vascular and pigmented lesions. *J Biomed Opt* (accepted) 2009.