Int J Thermophys (2010) 31:784–792
DOI 10.1007/s10765-010-0770-4

Optical Photoacoustic Detection of Circulating Melanoma Cells *In Vitro*

G. Gutiérrez-Juárez · S. K. Gupta · Ryan M. Weight · L. Polo-Parada · C. Papagiorgio · J. D. Bunch · J. A. Viator

Received: 26 June 2009 / Accepted: 27 May 2010 / Published online: 22 June 2010

Abstract The purpose of this research was to investigate the sensitivity of a system for the detection of circulating melanoma cells based on the thermoelastic properties of melanoma. The method employs photoacoustic (PA) excitation coupled with an optical transducer capable of determining the presence of cells within the circulating system in vitro. The transducer is based on stress wave-induced changes of the optical reflectance of a glass–water interface, probed with a continuous laser beam that is incident at an angle close to the critical angle of total internal reflection. A frequency tripled Nd:YAG laser pumping an optical parametric oscillator was employed to provide 532 nm and 620 nm laser light with a pulse duration of 10 ns. A custom-made flow chamber was used as an excitation and acoustic wave collection device. The targets were a human melanoma cell line HS 936 with an average diameter of about 15 μm. Melanoma cells were suspended in 10 mL of two types of media. The first

G. Gutiérrez-Juárez
Division de Ciencias e Ingenierias Campus Leon, Universidad de Guanajuato, Leon, Guanajuato 37150, Mexico

G. Gutiérrez-Juárez · S. K. Gupta · J. A. Viator (✉)
Department of Biological Engineering, University of Missouri, Columbia, MO 65211, USA
e-mail: viatorj@missouri.edu

J. A. Viator
Department of Dermatology, University of Missouri, Columbia, MO 65211, USA

L. Polo-Parada
Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO 65211, USA

L. Polo-Parada · C. Papagiorgio · J. D. Bunch
Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211, USA

R. M. Weight
Kansas City University of Medicine and Biosciences, Kansas City, MO, USA

one was Tyrode's buffer in concentrations ranging from 10 to 50 cells per μL, and the second one included 10^6 healthy white blood cells per mL of Tyrode's buffer. PA pressure waves were detected by an optical stress transducer. Detection trials resulted in a detection threshold of the order of one individual cell, indicating the effectiveness of the proposed mechanism. Results imply the potential to assay simple blood samples, from healthy and metastatic patients, to test the presence of cancerous melanoma providing an unprecedented method for screening metastatic disease.

Keywords Cancer · Laser-induced ultrasound · Melanoma cells · Nd:YAG laser radiation · Optoacoustics · Photoacoustic effect

1 Introduction

The main challenge for patients with completely removed primary tumors largely depends on the question whether distant metastasis will eventually arise from occult micrometastatic cells that were missed by initial tumor staging procedures. Metastasis is a cascade of linked sequential steps involving multiple host-tumor interacting cancer cells possessing multiple genetic abnormalities that grow unregulated and eventually lose the ability to adhere to one another [1] resulting in the spread of free malignant cells. These cells can easily migrate through the tumor membrane and extracellular matrix and travel to new sites via the lymphatic or blood systems [1]. Currently, the most common procedure for the detection of disseminating tumor cells is assaying lymph nodes or bone marrow at the time of diagnosis and surgery [2]. Histochemical examinations involving the staining of tissues using dyes such as hematoxylin and eosin can be used to track the short-term spread of cells through the lymphatic system [1]. However, these methods have proven inaccurate, time consuming, and painful [1]. In addition, 15 % of patients shown to be negative for lymphatic transport of metastases will develop metastatic disease in the future [1]. This occurs either by the lack of efficient detection by current techniques or by metastatic spread directly into the blood stream. Fisher et al. [3] have shown that metastatic disease has the ability to bypass the lymphatic system in its entirety and that the circulatory system may play a larger role in the spread of metastatic disease than that of the lymphatic system. Disseminating cells have been shown to be present in circulation in extremely low concentrations, estimated to be in the range of one tumor cell in the background of 10^6 to 10^7 normal blood cells [4]. Considering that Gusterson and Ott [5] calculated that a pathologist has a 1 % chance of identifying a metastatic focus in a patient with breast cancer, that is, three cells in diameter: it is not surprising that regions of metastasis within lymph nodes or other areas of the body often go undetected. Consequently, a technique is desired that can accurately detect the circulation of tumor cells of the order of 1 in 10^6 blood constituent cells with relative ease.

Recently, a system for the detection of melanoma circulating tumor cells has been reported [6]. This method employs photoacoustic (PA) excitation coupled with a detection system based on a PVDF transducer, and capable of determining the presence of disseminating cells within the circulatory system in vitro. The trials conducted consisted of the detection of dyed latex microspheres and a human melanoma cell

line. However, even when the sensitivity of the PA method employed is high (detection threshold of the order of 20 individual cells), it is insufficient for the concentration of disseminating cells. The experiments were based on the fact that most melanomas, being derived from melanocytes, contain some amount of melanin. Melanin is a strong optical absorber at all visible light wavelengths [7–9] and some lines of melanoma cells may contain a greater amount of them, since they show decreased binding and transfer of melanin to keratinocytes [10], enhancing the ability of laser light to create an acoustic signal.

In recent research, it has been shown that, using an optical detector for the PA waves, it is possible to detect one melanoma cell suspended in saline [11]. In this work, a variant of the PA wave detection is proposed. This variant is based on stress wave-induced changes of the optical reflectance of a glass–water interface probed with a continuous laser beam that is incident at an angle close to the critical angle of total internal reflection [12]. The sensitivity of a PA flow system for the detection of circulating melanoma cells based on the thermoelastic properties of melanoma is investigated. The method employs PA excitation coupled with an optical transducer capable of determining the presence of one melanoma cell within the circulating system in vitro.

2 Materials and Methods

2.1 Cancer Cells

Human malignant melanoma cell line HS 936 (ATCC) was cultured in suspension with RPMI-1640 growth medium (Sigma) + 10 % FBS, incubated at 37 °C, and suspended in 10 mL of Tyrode's buffer (125 mM NaCl, 4.7 mM KCl, 1.4 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 10 mM D-glucose, pH 7.4), resulting in two concentrations, 1 melanoma cell in 10 μL and 100 melanoma cells per μL. White blood cells (WBC) from a healthy volunteer were suspended in 10 mL of Tyrode's solution, in which one melanoma cell was suspended.

The diameter of the melanoma cells was determined by transmitted light microscopy and an immunostaining process and was about 15 μm. The immunostaining process is as follows: melanoma cells were cultured for 3 days in a thin 25 mm microscope cover glass (Fisher brand, Fisher Scientific) fixed with 3.7 % formaldehyde (Sigma-Aldrich, St. Louis, MO) for 10 min, washed five times for 5 min with PBS. Immunostaining was performed by blocking the sections with 2 % bovine serum albumin (BSA) in PBS and incubating with the appropriate primary antibodies (Tyrosinase-ab738, Abcam), at 4 °C for 24 h, followed by incubation with fluorochrome-conjugated secondary antibodies (Zymed, San Francisco, CA) for 2 h at room temperature, washing in PBS, and finally putting coverslips on the slides with Prolog Antifade (molecular probes). Images were digitally photographed at 40× magnification with an upright BX51WI Olympus (Tokyo, Japan) microscope equipped with an Axiocam MRC. Figure 1 shows pictures of human malignant melanoma cell line HS 936.T. A and B are transmitted light microscopy images of two different cell cultures, and C and D are immunofluorescence images of the same cells stained for tyrosinase. Note that even when the cells are not dark under the transmitted light (Fig. 1a and b), they express more tyrosinase (arrows in Fig. 1ac versus Fig. 1bd).

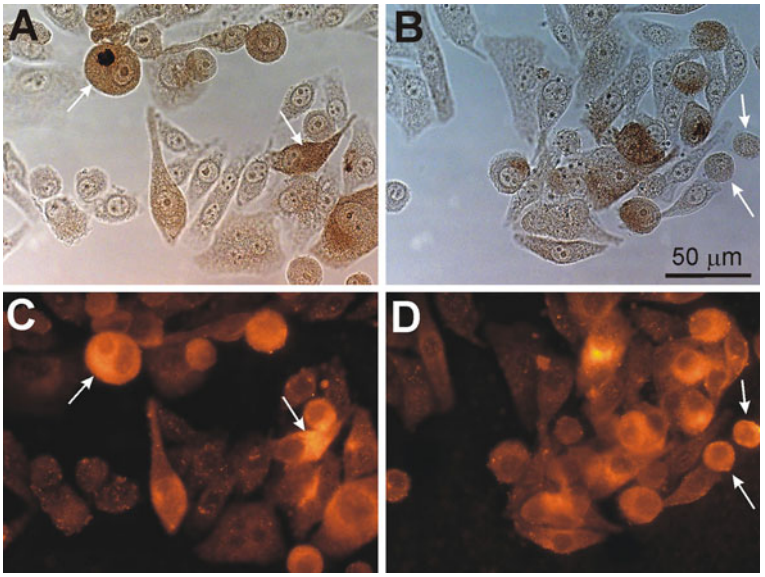

Fig. 1 Pictures of human malignant melanoma cell line HS 936.T. (**a**) and (**b**) are transmitted light microscopy images of two different cell cultures. (**c**) and (**d**) are immunofluorescence of the same cells stained for tyrosinase. Note that the darker cells under transmitted light (**a**) and (**b**) do not represent the cells that express the most tyrosinase (*arrows* in (**ac**) versus (**bd**))

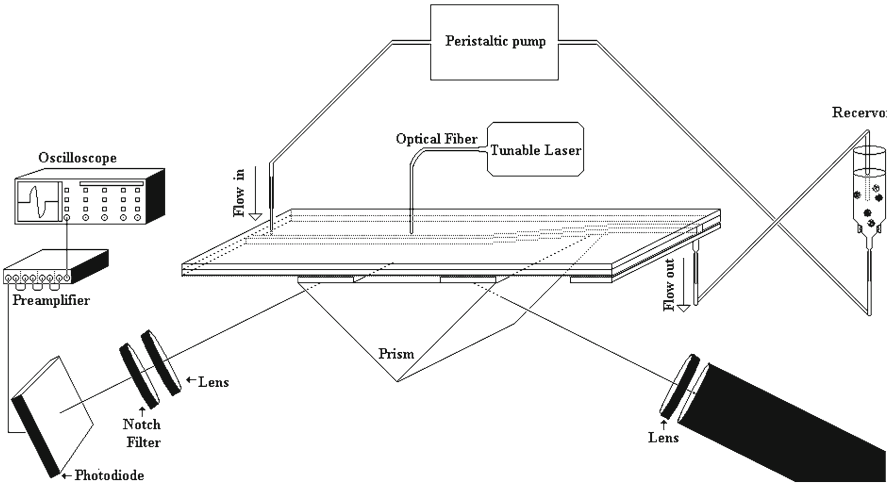

Fig. 2 Schematic representation of photoacoustic circulating detection setup

2.2 Experimental Setup

The experimental setup for the PA detection of metastatic melanoma cells appears in Fig. 2. A frequency tripled Nd:YAG laser pumping an optical parametric oscillator (Vibrant 355 II, Opotek. Carlsbad, CA, USA) was employed to provide 532 nm and

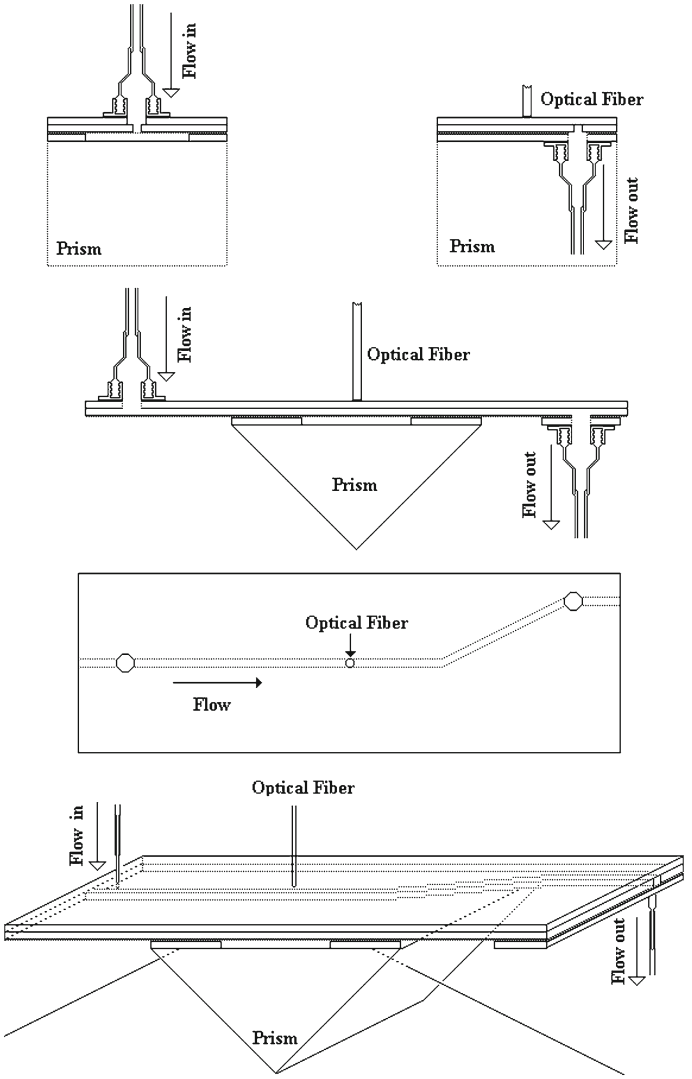

Fig. 3 Photoacoustic cell, including irradiated microparticles and prism where the reflection of Fresnell occurs

680 nm laser light with a pulse duration of 5 ns. Beam energy entering the flow cell was 6.8 mJ, with a pulse repetition rate of 10 Hz. The pulsed light beam was delivered via an optical fiber 1 mm in diameter. Radiant exposures were $0.395\,\mathrm{J}\cdot\mathrm{cm}^{-2}$ with a spot size of 1 mm of radius. A peristaltic pump (Masterflex L/S Economy Drive, Cole-Parmer Instruments) and platinum-cured silicon tubing (L/S 14, Cole-Parmer Instruments) facilitated the circulation of experimental solutions. A custom-made flow chamber was used as an excitation and acoustic wave collection device (Fig. 3). Solutions were circulated at a rate of $0.23\,\mathrm{mL}\cdot\mathrm{s}^{-1}$ for approximately 2 min. Acoustic pressure waves were detected by an optical stress transducer (Fig. 3) attached to the flow chamber.

The design of the optical stress transducer used in our experiments is shown in Fig. 2. The HeNe laser beam is reflected at the surface of a right angle glass prism. A lens with a focal length of 120 mm is used to focus the beam to an elliptical spot on the glass–water interface. A second lens images the HeNe laser spot on the prism surface onto the active area of the photodiode (Si 1 GHz photo-receiver, 1601-FS-AC, New Focus). In the experiments, the opposite side of the sample facing the glass prism was irradiated by the laser pulses. In this case, the wave arrives at the detector after passing through the sample (transmission mode). The targeted cells and resulting PA signals were detected by an optical stress transducer and converted into a voltage signal displayed by a 200 MHz oscilloscope (TDS 2024, Tektronix, Wilsonville, OR, USA) triggered by a photodiode. The signals were amplified with a gain of 25 via a 350 MHz amplifier (SR445A Stanford Research Systems).

The flow chamber or PA chamber (Fig. 3) was made as follows: a hole was formed in one end of a glass microscope slide using a spherical grinding bit with a diameter of about 4.9 mm. The hole was located about 5 mm from the end of the slide and as close to the center line as possible. Another slide was cut using a glass cutter along the long axis into two pieces similar to the figure above. The parallel cut in reference to the sides was in the center and ran about 2/3 the length of the slide. Another cut was run from the end of the parallel cut diagonally to roughly the corner of the slide. A straight edge device was used when scoring the glass to make sure the score is straight. Then, a cylindrical grinding bit with a diameter of about 4.9 mm was used to grind holes in the diagonal cut of the two cut glass pieces. These holes were placed about 4.9 mm from the corner end of the diagonal cut. Then, silicone sealant was used to adhere the three pieces together as shown in Fig. 3. The silicone was placed on the two cut pieces which were then placed on the other glass slide so that there was a 1 mm space between the two cut pieces. Plastic wrap was adhered to the other side of the two cut pieces using silicone with a similar procedure as described previously. With this procedure, we formed a channel with a cross section of $1\,\text{mm}^2$. Then, a $10\,\text{mm} \times 25\,\text{mm}$ piece of glass slide was adhered to either end on the plastic wrap side in order to reinforce the plastic wrap around the holes. A hole was ground in the 12.7 mm piece of glass that covers the hole in the diagonal section so that the two are aligned. This hole had to be ground prior to being attached. A spherical grinding bit with a diameter of 6 mm was also used for this hole. Then, a band saw was used to cut off the end where the needle attaches of two 3 mL syringes. Using silicone, these ends were then attached to the slide so that they covered the open holes. Therefore, one end is on the top side of the slide and the other end is on the bottom side of the slide on the opposite end.

3 Results and Discussion

Cultured live human melanoma cells in 10 mL of Tyrode's buffer were introduced into the flow system and irradiated with 6.8 mJ of incident light energy. Figure 4a shows the PA waveforms from 532 nm and 622 nm laser irradiation. The two signals were artificially offset by 10 mV for comparison. Irradiation of a pure Tyrode buffer showed

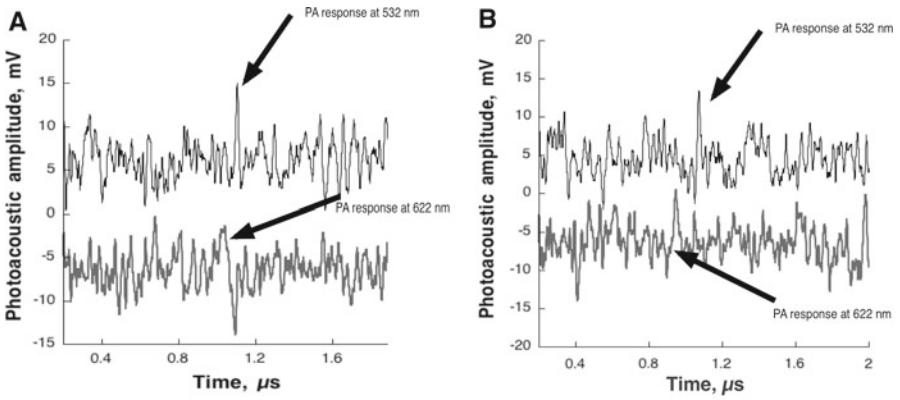

Fig. 4 Photoacoustic waveform for the Tyrode's buffer and the lowest concentrations detected of human melanoma cell line HS 936: (**a**) one melanoma cell per milliliter of Tyrode's buffer and (**b**) one melanoma cell per microliter

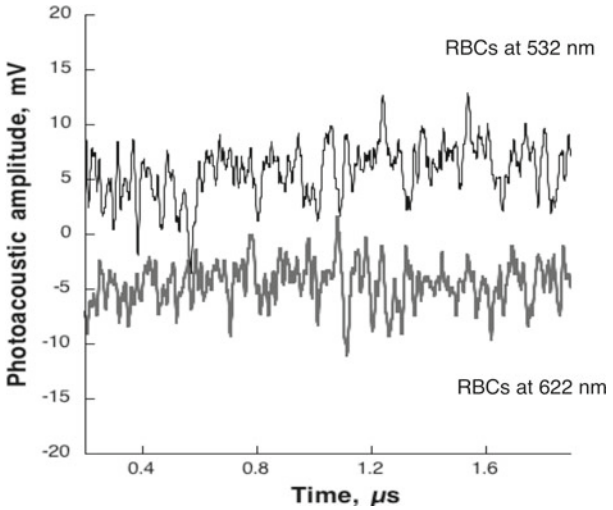

Fig. 5 Photoacoustic waveform for Tyrode's buffer and the low concentration of red blood cells in Tyrode's solution

no PA events and had a root mean square (RMS) of about 2 mV. The PA responses shown in this figure showed greater discrimination in real time, more so than in the static view offered by the figure. As each oscilloscope trace was triggered by the laser pulse at 10 Hz, random fluctuations changed at that rate. However, particles in flow showed a persistent, though slowly changing signal among the random fluctuations. As the signals were averaged 16 times on the oscilloscope at a refresh rate of 10 Hz, the flow rate was slow enough that any absorbing particles were shown as slowly evolving signals over a period of 1.5 s to 2.0 s. This time resolved contrast made detection of absorbing particles more obvious than otherwise shown by the signal-to-noise

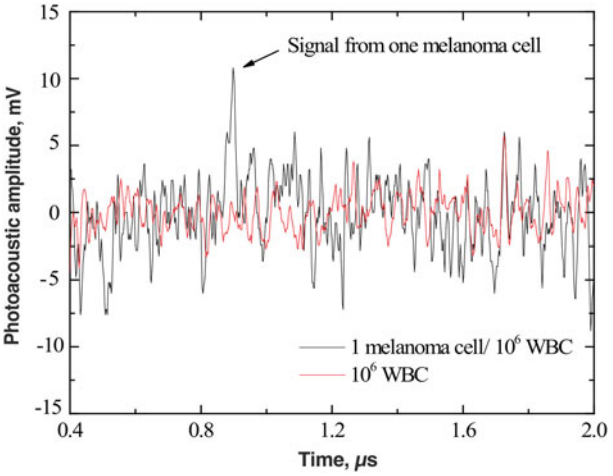

Fig. 6 PA waveform for two melanoma cells in white blood cells

ratio (SNR). The signals shown in Figs. 4–6 were identified by the SNR as well as by observing this signal persistence. Thus, similar spikes in each figure were discounted as absorbing particles due to their much shorter persistence of 100 ms, corresponding to the 10 Hz triggering of the oscilloscope.

Figure 4b shows similar waveforms for one cell per microliter at 532 nm and 622 nm. For samples with melanoma cells, a PA signal of about 1.1 μs is observed. The geometry of the PA chamber and optical detection device were conducive to this time domain following the initial laser pulse due to a 1.5 mm per μs acoustic wave propagation rate in water. Thus, these results accurately reflect the expected melanoma signal. The buffer solution experiment was run in order to absolve results of false positives due to absorption by the buffer medium.

In order to absolve results of false positives due to absorption by red blood cells (RBC), sample compost of five human RBCs per microliter of Tyrode's buffer was introduced in the same conditions that melanoma exists in Tyrode's solution. The RMS of this signal was also about 2 mV. These RBCs created much smaller PA signals than the melanoma cells and were not immediately obvious, as shown in Fig. 5. There were signals that persisted for 1.5 s to 2.0 s, although their amplitudes were about equal to 10 Hz random fluctuations, as shown in Fig. 5.

A most remarkable fact is that optical detection is able to show that WBCs give no signal and that we can detect dilute suspensions of melanoma cells. In order to test this effect, one single human melanoma cell was separated and diluted in 10^6 WBCs in 10 mL of Tyrode's solution that was introduced into the flow system and irradiated with 6.8 mJ of incident light energy at 532 nm, resulting in a large PA signal between 0.8 μs and 0.9 μs. With a noise level of 2 mV, the SNR of the melanoma signal was about 5:1. In Fig. 6, the continuous line with the sharp peak at about 0.9 μs corresponds to the melanoma cell, and the other one is for the WBC suspended in Tyrode's solution. As can been seen, the WBCs did not impede the ability to detect melanoma cells. This work was repeated five times in this evaluation.

4 Conclusions

It is shown that the PA detection system presented here has the ability to detect a single melanotic melanoma cell in vitro. The PA system allows detection of circulating tumor cells in patients with melanoma cancer. In addition, it is possible to increase the sensitivity by nanoparticles attached to the cancer cells, indicating the development of a reliable PA diagnostic test to aid clinicians in the management and treatment of patients.

The method described offers a tool for the enrichment of micrometastatic tumor cell clusters; these clusters may represent the initial stage of development from a single disseminated tumor cell toward an overt metastasis. Future studies will include clinical trials testing the PA ability to detect metastatic melanoma present in the blood stream of Stage IV metastatic patients suffering from multiple types of cancers. The possibility to amplify detection through the attachment of secondary molecules and antibodies will be looked at as well as adapting the method to include amelanotic tumor cells. It is our goal that this technology can eventually be used as an efficient and inexpensive method to routinely check for the presence of malignant circulating cancer cells. This work presents an innovative approach to solving a very complex, poorly understood area of medicine. The successful detection of individual metastatic cancer cells is an unprecedented achievement that bears direct clinical relevance with the hope of uncovering metastatic disease in its early stages, thus improving the prognosis of millions of cancer patients worldwide each year.

Acknowledgments We thank the CONACyT-Mexico, Universidad de Guanajuato-Mexico, and University of Missouri for their financial support during the sabbatical stay in the Life Science Center of the University of Missouri. This work was supported by NIH R21 CA 139186–02 and the Missouri Life Sciences Research Board. We also thank the American Society for Laser Medicine and Surgery and the Wallace H. Coulter Foundation for their financial support.

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