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Photoacoustic flow cytometry

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Abstract

Conventional flow cytometry using scattering and fluorescent detection methods has been a fundamental tool of biological discoveries for many years. Invasive extraction of cells from a living organism, however, may lead to changes in cell properties and prevents the long-term study of cells in their native environment. Here, we summarize recent advances of new generation flow cytometry for *in vivo* noninvasive label-free or targeted detection of cells in blood, lymph, bone, cerebral and plant vasculatures using photoacoustic (PA) detection techniques, multispectral high-pulse-repetition-rate lasers, tunable ultrasharp (up to 0.8 nm) rainbow plasmonic nanoprobe, positive and negative PA contrasts, *in vivo* magnetic enrichment, time-of-flight cell velocity measurement, PA spectral analysis, and integration of PA, photothermal (PT), fluorescent, and Raman methods. Unique applications of this tool are reviewed with a focus on ultrasensitive detection of normal blood cells at different functional states (e.g., apoptotic and necrotic) and rare abnormal cells including circulating tumor cells (CTCs), cancer stem cells, pathogens, clots, sickle cells as well as pharmacokinetics of nanoparticles, dyes, microbubbles and drug nanocarriers. Using this tool we discovered that palpation, biopsy, or surgery can enhance CTC release from primary tumors, increasing the risk of metastasis. The novel fluctuation flow cytometry provided the opportunity for the dynamic study of blood rheology including red blood cell aggregation and clot formation in different medical conditions (e.g., blood disorders, cancer, or surgery). Theranostics, as a combination of PA diagnosis and PT nanobubble-amplified multiplex therapy, was used for eradication of CTCs, purging of infected blood, and thrombolysis of clots using PA guidance to control therapy efficiency. *In vivo* flow cytometry using a portable fiber-based device can provide a breakthrough platform for early diagnosis of cancer, infection and cardiovascular disorders with a potential to inhibit, if not prevent, metastasis, sepsis, and strokes or heart attack by well-timed personalized therapy.

Keywords

In vivo flow cytometry; Photoacoustic spectroscopy; Negative contrasts; Blood and lymph flow; Cerebrospinal fluids; Circulating tumor cells; Clots; Sickle cells; Red blood cell aggregation; Ultrasharp plasmonic resonances

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1. Introduction

Flow cytometry is a well-established powerful analytical tool that has led to many revolutionary discoveries in cell biology and molecular disease diagnosis [1–2]. In conventional flow cytometry, cells are introduced into a high speed (up to few m/s) laminar artificial flow. After focusing the cells into a single file, laser-induced fluorescence, and/or forward and sideways scattered lights emitted from the cells are detected using photodetector arrays with spectral filters. This highly accurate technology provides fast (a few million cells in a minute), multiparameter quantification of the biological properties of individual cells at subcellular and molecular levels, including their functional states, morphology, composition, proliferation, and protein expression.

Nevertheless, invasive extraction of cells from a living system may alter cell properties (e.g., morphology or marker expression) and prevent the long-term study of cells (e.g., cell-to-cell interactions, aggregation or rolling) in their natural biological environment. Other limitations include low sensitivity for detection of rare circulating tumor cells (CTCs), bacteria, sickle cells and clots due to a small blood sample volume, and the discontinuity of sampling with limited, discrete time points. These shortcomings can be solved by the development of *in vivo* flow cytometry which allows noninvasive, continuous assessment of the large blood volume circulating in the blood vessels. However, the adaptation of flow cytometry principles from *in vitro* application with cells flowing in a well-controlled single file, to *in vivo* studies using the blood and lymph vessels as natural tubes with native cell flow faces many challenges. These include: (1) poor optical conditions such as absorption, scattering, and autofluorescent background from the vessel wall, surrounding tissue and bulk blood cells; (2) multiple-file cell flow in vessel cross-sections; (3) difficulties of accessing deep vessels; (4) problems with the use of a transillumination (forward) or sideways optical schemes; and (5) instability of blood, and especially, lymph-flow parameters (e.g., fluctuation of cell velocity and the positions of cells in vessel cross-sections). These limitations require some precautions in the choices of a vessel location, detection system, and proper animal models as a first step toward transitioning this technique to human applications.

A brief history, features and challenges of this new generation of flow cytometry using photothermal (PT), photoacoustic (PA), fluorescence, transmission, and Raman detection methods were recently reviewed with focus on previous work in this field before 2009 [3]. In particular, the first pioneer's efforts resulting in the development of *in vivo* flow cytometry with PT, PA, and scattering detection techniques [4–23], were summarized in book chapters [24–26]. It included real-time detection of circulating red and white blood cells (RBCs and WBCs respectively) in different functional states (e.g., normal and apoptotic), CTCs (squamous carcinoma), bacteria (*Escherichia coli* and *Staphylococcus aureus*), nanoparticles (NPs) (e.g., gold nanorods [GNRs], carbon nanotubes [CNTs], and magnetic NPs [MNPs], dyes (e.g., Lymphazurin, Evans blue, and Indocyanine Green [ICG]), the use of lymph valves as natural nozzles to focus cells into a single file *in vivo*, minimally invasive flow cytometry with fiber inside vessels [30], and *in vitro* PA flow cytometry (PAFC) [30,33] for verification of PA data *in vivo*.

Here, we summarize recent advances of PAFC platform and its new applications [27–42]. The technical PAFC's improvements include high-pulse repetition-rate lasers, fast signal acquisition algorithms, time-of-flight measurement of cell velocities, focused ultrasound transducers for the assessment of deep vessels, ultrasharp rainbow NPs as high-contrast molecular PA and PT theranostic nanoprobe with super-narrow tunable spectral PA and PT resonances (up to 0.8 nm), *in vivo* high speed image flow cytometry of cells of interest at velocities up to 2 m/s, *in vivo* fluctuation flow cytometry, integration of PAFC and fluorescent cytometry with positive and negative contrast modes, and the combination of PA diagnosis and PT therapy (PA-PT nano-theranostics). The new applications include: (1) label-free monitoring of melanoma CTCs released during palpation, biopsy, and conventional and laser surgery; (2) multiplex targeting, magnetic enrichment, and detection of breast bulk and stem CTCs; (3) PA detection of CTCs (called also disseminated tumor cells) in lymphatics as the earliest prognostic marker of metastasis compared to sentinel lymph node and blood assessment; (4) targeted detection of pathogens within the bloodstream at a single bacteria level; (5) PA blood rheology including real-time monitoring of RBC aggregation; (6) identification of sickle cells; (7) study of cell death in blood circulation; (8) study of pharmacokinetics of NPs, liposomes, dyes and other contrast agents including their dynamic interaction with blood cells; (9) cell and NP identification based on their different velocities in blood flow; (10) theranostics as real-time, PAFC-guided, PT elimination of CTCs and bacteria from circulation [30], and (11) a minimally-invasive delivery of laser radiation to deep vessels inside the body using a fiber in a tiny needle or catheter.

2. Principle of *in vivo* flow cytometry with positive and negative contrasts

2.1. General schematics

The underlying principle of *in vivo* flow cytometry with a PA detection schematic (referred to as PAFC) is based on the irradiation of selected vessels using short laser pulses followed by time-resolved detection of laser-induced acoustic waves (referred to as PA signals) with an ultrasound transducer gently held against the skin (Fig. 1A, right). The physical mechanism of PAFC is based upon the PA effect associated with fast (picosecond scale) nonradiative relaxation of absorbed laser energy into heat and then the thermoelastic generation of acoustic waves [43–49]. PAFC as a combination of laser and ultrasound techniques has a high sensitivity and spectral specificity of optical methods and a high spatial resolution and depth penetration of ultrasound methods. Fig. 1B illustrates a typical PA signal trace that occurred during label-free detection of melanoma CTCs (B16F10) on the tumor-bearing mouse model. Each individual peak in this trace obtained by averaging many PA signals (20–100) is associated with individual single or aggregated CTCs.

Most studies were performed on well-distinguished, 30–70- μm diameter blood vessels located approximately 40–100 μm deep in the thin ear ($\sim 250 \mu\text{m}$) of the nude mouse model, and on 200–300- μm diameter blood vessels in the abdominal area of the nude mouse at a depth of 0.3–0.5 mm, or on the aorta (0.9–1 mm) at a depth of 2–4 mm [30]. The mice were anesthetized using a standardized procedure and placed on a heated microscope stage with the ear spread flat over a glass slide. The ultrasound transducer was acoustically connected

to the mouse's ear using warm water, or conventional ultrasound gel, topically applied on the biotissue. In particular, the transducer can be placed in a small cylindrical water tank with a flexible transparent membrane attached to skin.

Laser radiation can be delivered to biotissue either by using a microscope schematic with a customized condenser to create the desired linear beam shapes (e.g., from 5×50 to 25×150 μm), or a fiber with a miniature tip and cylindrical optics. PAFC offers the highest absorption sensitivity at the single-cell level which allows noninvasive (a temperature elevation 0.1°C) detection and imaging of individual cells. PAFC molecular specificity is provided either by label-free intrinsic absorption spectroscopic contrast (e.g., from hemoglobin [Hb], melanin or cytochromes [Cyt]), or by strongly absorbing, low-toxicity, functionalized NPs. The spatial resolution at the level of $5\text{--}10$ μm in superficial $30\text{--}70$ μm in diameter vessels at a depth of $0.1\text{--}0.5$ mm is determined by optical focal parameters, while in deeper tissue with strong light scattering, the resolution depends upon ultrasonic focal parameters (e.g., $100\text{--}20$ μm at a frequency of $10\text{--}50$ MHz, respectively). The focused cylindrical ultrasound transducer provides a minimal detected volume due to high lateral resolution with simultaneous assessment the entire cross section of a vessel. The attenuation of ultrasound waves at typical frequencies of $5\text{--}30$ MHz is almost negligible (~ 0.6 dB cm^{-1} MHz $^{-1}$) for depths of $1\text{--}5$ mm [46,47]. Indeed, the PA technique demonstrated an advantage in assessing deep vessels *in vivo* at typical levels of $0.5\text{--}1$ cm with a few studies at $5\text{--}7$ cm, compared to other optical modalities [46].

2.2. Lasers, transducers and signal acquisition algorithm

To expose each fast moving cell, the laser pulse rate, f , should be $f \sim 1/t_L$, where $t_L = (D + d)/V_F$ is the lifetime of the moving cell in the detection volume, D is the cell diameter, d is the width of the linear laser beam, and V_F is the velocity of the cell [15]. For $D \approx d \approx 10$ μm , in vessels with diameters of 50 μm , 2 mm (e.g., hand vein), and 1 cm (e.g., carotid artery) having different velocities pulse rates will be $f \sim 500$ Hz, 2 kHz, and 50 kHz, respectively. To improve signal-to-noise ratios (SNRs) by averaging PA signals from each exposed cell without temporal overlapping of thermal effects, f can be increased until $f \sim 1/\tau_T$, where $\tau_T = D^2/28k$ is the thermal relaxation time (for a spherical target with diameter of D), and k the thermal diffusion [39]. For $D = 10$ μm and $k = 1.4 \times 10^{-7}$ m^2/s (for water), $f \sim 25$ kHz. The number of PA signals from one cell, N , can be estimated from the equation $N = f \times t_L$ (e.g., $N = 40$ for $V_F = 5$ mm/s and $f = 10$ kHz). The effective generation of PA signals requires acoustic confinement for a laser pulse duration $t_p \sim D/c_s$ [44–46], where c_s is the speed of sound. For a typical cell size of $10\text{--}15$ μm and $c_s = 1.5 \times 10^5$ cm/s (for water), $t_p \sim 10$ ns. For smaller targets, such as single melanin or plasmonic NPs with sizes of $30\text{--}100$ nm $t_p \sim 50\text{--}500$ ps.

Our setup was equipped with five high-pulse-repetition-rate nanosecond lasers with the following parameters: (1) wavelength, 532 nm; pulse energy, 100 μJ ; pulse width, 5 ns; and repetitions rate, up to 100 kHz (model: LUCE 532, Bright Solutions, Italy); (2) 671 nm, 35 μJ , 25 ns, and 100 kHz (model: QL671-500, CrystaLaser, USA); (3) adjustable spectral range, $720\text{--}890$ nm (820 nm used in most studies); 75 μJ , 8 ns, and 30 kHz (model: LUCE 820, Bright Solutions); (4) 904 nm, 5 μJ , 15 ns, 10 kHz (diode laser, model 905-FD1S3J08S,

Frankfurt Laser Company); and 5) 1064 nm, 100 μ J, 10 ns, and 1–750 kHz (model: MOPA-M-10, Multiwave Photonics, Portugal). The laser beams were navigated on selected blood vessels with an X–Y motorized stage using a controller (STG4400ML, Conix Research, Inc., Springfield, OR) under the optical imaging guidance. In most applications, the pulse-repetition rate for these lasers was 10 kHz and in selected studies, up to 500 kHz [33]. Laser pulses were triggered by a digital delay/pulse generator (DG645, Stanford Research Systems, USA). The selected contrast agents (e.g., NPs, or dyes) displayed peaks in the absorption spectra coinciding with the laser wavelength used (e.g., 532, 671, 820, 904, and 1064 nm).

The PA signals had a bipolar shape with a typical duration of 0.1–1 μ s. They were usually transformed into a short pulse train (Fig. 2A, top right) because of the reflection and resonance effects in the transducer holder. PA signals were detected by ultrasound transducers (e.g., unfocused: model 6528101, 3.5 MHz, 5.5 mm in diameter, Imasonic Inc., Besançon, France; and focused: model V316-SM, 20 MHz, focal length 12.5 mm, Panametrics) and then amplified (amplifier models: 5662, bandwidth 50 kHz–5 MHz, gain 54 dB; and 5678, 40 MHz, gain 60 dB, both from Panametrics-NDT, Olympus). To collect PA signals, the PAFC setups were equipped with a high-speed, analog-to-digital converter boards (e.g., National Instruments Corp.). In analogy to conventional flow cytometry, final PA data were presented as PA signal traces (Fig. 1B) which were analyzed using customized software. A real-time and post-processing operations were performed using MATLAB. The customized peak analyzer identifies the baseline in the trace, sets the threshold level based on the mean and a multiple of the standard deviation, selects the peaks above this threshold, and finally performs statistical analysis by acquiring time stamps, amplitudes, and widths of the detected peaks.

2.3. Combination of photoacoustic, fluorescent, and photothermal methods with positive and negative contrast modes

The use of a single technique limits the range of detectable cells with different optical properties. To increase the number of detectable cells, conventional flow cytometry usually integrates fluorescent, and scattering detection methods. In 2005 we proposed the integration of PT, PA, fluorescence and scattering detection methods for *in vivo* flow cytometry [9,10] which were previously used separately only [3–40,50–54]. Recently, we demonstrated advances of real-time integration of PAFC and fluorescent flow cytometry (FFC) termed as PAFFC [42] using pulsed and continuous wave (CW) lasers as traditional sources for the generation of PA and fluorescent signals, respectively (Fig. 1A). In addition, we showed the opportunity to use pulsed lasers only for the simultaneous generation of both signals that simplified the PAFFC schematic and provided time-resolved discrimination of objects with different lifetimes (e.g., quantum dots with long fluorescence compared to blood that has a shorter autofluorescence background).

Conventional PA and fluorescence techniques use preferential positive imaging contrasts when signals from strongly absorbing or fluorescent cells are above absorption or autofluorescence background, respectively. In particular, laser irradiation of blood vessels creates a constant PA background associated with absorption of the high number of RBCs in

the irradiated volume. Thus, to be detected using the positive contrast mode, targets must have higher absorption compared to the RBC background as in the case of pigmented melanoma cells [30] or cells targeted by strongly absorbing NPs [28]. In PAFFC, conventional positive contrast mode was combined with the new negative PA and fluorescent contrast modes, in particular, for PA detection of cells having lower absorption than RBCs as in case of platelets or WBCs (Fig. 2B). When weakly absorbing (e.g., platelet rich white clot, Fig. 2A) or low fluorescent cells pass through laser-irradiated vessel volume, a transient decrease of the absorption or fluorescence results in a sharp negative PA and fluorescent peaks in blood absorption and the fluorescence background, respectively (Fig. 2C, middle). Thus, absorption and autofluorescence background as a problem in conventional positive contrast PAFC and FFC is transformed to an advantage using negative contrast PAFC, FFC, and especially its combination in PAFFC. The negative contrast can be used for label-free counting of RBCs, WBCs, and red (with dominant RBC content), white (with dominant platelet content), and combined clots (Fig. 2C, right) (see Section 6). Negative contrasts of cells can be enhanced either by their targeting with negative PA and fluorescent probes (e.g., weakly absorbing or fluorescent beads, respectively), or by increasing background through the injection of absorbing or fluorescent contrast agents. The appearance of many small negative and positive contrast objects in the detected volume, such as mixed red-white clots or RBC aggregates leads to the generation of the fluctuated PA background which is the primary subject of *in vivo* fluctuation flow cytometry (Fig. 2D) (Section 6).

PAFC was also integrated with a PT flow cytometry (PTFC) [10,14] which was also used for verification of PA data. In the PT imaging mode [8–10,55,56], irradiation (e.g., using tunable optical parametric oscillator with wavelength of 415–2300 nm, pulse width of 8 ns, pulse rate of 100 Hz and pulse energy of 0.1–500 μ J) of absorbing objects leads to temperature-dependent variations of the refractive index that are visualized with phase-contrast [55] or a multichannel thermal lens schematic [57] using a second, collinear laser probe pulse from a Raman shifter (639 nm, 12 ns, 0.01–10 μ J) and a CCD camera. In the PT single-channel thermal lens mode, a laser-induced refractive heterogeneity causes defocusing of a collinear He-Ne laser probe beam (633 nm; power, 1.4 mW) and hence a reduction in the beam's intensity at its center, as detected by a photodiode with a pinhole (referred to as PT signals). PT signals from single cells in a linear mode (i.e., without notable cell photodamage) represent a standard positive peak associated with rapid (pico-nanosecond scale) cell heating and a slower, microsecond scale tail corresponding to cell cooling (Fig. 2A, bottom, right). An advantage of the PAFC is its backward mode (i.e., laser and transducer are on one side), while PTFC has better sensitivity in transillumination mode. PT and PA methods beneficially supplement each other, and in combination, provide a very powerful diagnostic and therapeutic tool. For example, non-invasive PA diagnostics can be integrated with PT killing of metastatic or residual cancer cells, either static or in flow, using more powerful laser pulses triggered by PA signals from these cells.

2.4. Labeling in vivo

The great advantage of *in vivo* flow cytometry is the possibility for cell detection without labeling, in particular, by using the positive and negative PA contrast of RBCs and WBCs,

respectively, vs. the negative and positive fluorescent contrast of the same cells [42]. In this scenario, PA (or PT) signals can be generated using intrinsic chromophores and pigments such as hemoglobin, melanin, cytochromes, or carotenoids. Cells with a low endogenous absorption can be labeled directly in the bloodstream through intravenous injection of strongly absorbing functionalized NPs [28,37,40,42]. Depending upon cell and NP properties, the labeling procedure using mouse models takes from 10–20 min to one hour. High labeling specificity is provided through the selection of molecular markers that are highly expressed in targeted cells (e.g., CTCs), but almost absent in normal blood and endothelial cells (e.g., folates in CTCs) [28,51]. High labeling efficiency is associated with frequent NP-CTC collisions in partly turbulent blood flow. In accordance with our modeling, injection of 10^{10} NPs into the mouse's blood circulation with a volume of ~2 mL provides, on average, $\sim 10^3$ NP-CTC collisions per min with expected differences in the velocities of NPs and CTCs 1 mm/s, while their absolute velocities may be 5–10 mm/s. This allows the capture of antibodies by cell surface markers, and the capturing efficiency does not decrease at relative differences in the velocities of NPs and cells at 1–1.5 mm/s and the shear stress 0.5 dyn/cm² [58]. The PA signals from targeted cells with a typical NP number, ranging from 500–5000 NPs per CTC is much higher than the PA background from RBCs, unbound NPs with typical numbers of 1–10 in the detected volume, or from NPs nonspecifically bound to normal blood cells (e.g., macrophages). NP clustering around naturally densely packed cancer markers leads to significant enhancement in PA signals (at least 5–10-fold), and a red-shift effect in the absorption of coupled NPs in clusters [59], both of which serve as indicators of successful cell targeting. In most studies, we optimized the amount of injected NPs which range from 10^9 – 10^{10} NPs per mouse. These NPs did not produce notable signals immediately after injection, but a latter gradual increase in the PA signal amplitude and rate indicated a successful labeling process. Occasionally, strong PA signals can be observed immediately after intravenous injection of NPs over the period of few minutes, which is associated with NP aggregates which then are quickly (typically within few minutes) cleared from the circulation. To minimize this effect, before the injection, NP clusters are disaggregated by ultrasound and then filtered. Nevertheless, if short lasting signals from NP alone occur immediately after injection, these signals can be differentiated from signals related to cell targeting *in vivo* because the latter appear typically after a time delay.

3. *In vivo* real-time detection of circulating tumor cells

Most cancer deaths (up to 90%) are related to metastasis in the distant organs due to the hematogenous dissemination of CTCs shed from the primary tumor. Therefore, enumeration of CTCs appears to be a prognostic marker of metastatic development (the lower the CTC count, the longer the survival), cancer recurrence, and therapeutic efficacy [58]. However, incurable metastases can be developed at the time of the initial diagnosis with exiting CTC assays [58] which the current sensitivity threshold of 1–5 CTC/mL (i.e., 5,000–50,000 CTCs in the entire adult blood volume of ~5 L) is limited by the small sample volume (5–10 mL) obtained from the patient. The sensitivity threshold can be improved by assessment of a significantly larger blood volume up to the patient's entire blood volume using *in vivo* PAFC. We demonstrated that PAFC has the potential for label-free detection of melanoma

CTCs [30], a multiplex targeted detection of breast CTCs using advanced NPs [28], magnetic enrichment of CTCs *in vivo*, estimation of the efficiency of PT eradication of CTCs, and study of the impact of different interventions on CTC counts.

3.1. Label-free detection of circulating melanoma cells during tumor progression

We selected cutaneous melanoma as an almost ideal model for PAFC technology to provide routine, label-free, *in vivo* clinical assessment of CTCs for earlier detection of the most aggressive and epidemically growing malignancy which often progress to incurable metastasis at a very early stage of the disease. The label-free nature of PAFC when applied to melanoma denotes that PACF can be translated to clinical application much sooner for melanoma than for other cancers, with obvious and beneficial public-health consequences for this devastating disease. In this study, the over-expression of melanin was used as an intrinsic melanoma cell marker, which provides high PA contrast in the near-infrared (NIR) range in the blood background. Real-time PA counting of metastatic melanoma CTCs (B16F10) was performed in 50 μm -diameter ear mouse vessels, 200- μm abdominal vessels, and the 0.9-mm aorta (with focused ultrasound transducer) during tumor progression in the ear and skin of the nude mouse model (Fig. 3A,B). The CTC rates in these vessels were: 0.05, 2.7, and 91 CTCs/min, respectively (Fig. 3C), underscoring the higher probability of detecting CTCs in larger vessels with higher flow rates, particular the aorta, through which nearly the entire blood volume of a mouse ($\sim 2\text{ mL}$) circulates within 0.5–1 min compared with many hours (up to two days) in the smaller ear vessels. It should be emphasized that cell flow rate even in the mouse aorta is in the range of 10^7 – 10^8 cells/s which is 10^2 – 10^3 times higher than those achieved with modern conventional flow cytometry *in vitro*.

Scanning of a focused laser beam in the vicinity of the primary tumor in the mouse ear (PA scanning cytometry mode) revealed local PA signals from migrating individual or clustered melanoma cells in the first week after tumor inoculation. Metastatic cells appeared in ear microvessels near the tumor on week one with almost no cells detected in the abdominal skin blood vessels. Later, CTCs appeared in the systemic circulation. This indicates a much greater likelihood of detecting the initial metastatic process in the vicinity of the primary tumor before CTCs are disseminated in the large blood pool. The skin tumor growth rate was faster than that of ear tumors, and CTCs also appeared more quickly in the circulation. In particular, by week one, 1–4 CTCs/min were detected in the skin vasculature, and as the tumor size increased, the number of CTCs gradually increased (Fig. 3D) to ~ 7 and ~ 12 CTCs/min by 3 and 4 weeks, respectively. On the occasion, either PA signals with complex shapes, or one large PA signal were observed which support the hypothesis of circulating melanoma cells as aggregates. Indeed, optical imaging of ear vessels near a tumor revealed CTC aggregates on the vessel wall, indicating a high probability of CTC aggregating during intravasation.

The mice were euthanized, and tissue sections from different organs (e.g., lung, liver, brain, and lymph nodes) were examined by immunohistochemical staining (see details in [30]). No evidence of metastasis was found during the first 3 weeks after tumor inoculation for the ear tumor model, while PAFC demonstrated early detection of CTCs 4 days after tumor

inoculation [30,37]. Thus, CTCs can be readily detected with PAFC weeks before any evidence of detectable with conventional techniques metastasis.

By PA counting rare CTCs in the aorta we estimated PAFC's sensitivity threshold as 0.5–1 CTCs/mL that was verified *ex-vivo* by assessing whole blood volume with scanning PT and PA cytometry [30]. This unprecedented threshold sensitivity on the animal model provides an opportunity to use PAFC as a powerful research tool to study CTC behaviors and CTC's role in metastasis development at an early cancer stage. The PAFC sensitivity has a potential to be further improved 1000-fold (i.e. ~1 CTC/1000 mL) by the examination of a larger blood volume in humans, which is unachievable using existing assays. We recently developed a portable clinical prototype of PAFC using a high-pulse-repetition rate laser at 1064 nm with pulse rates up to 0.5 MHz, fiber delivery of laser radiation, and a focused, ultrasound transducer gently attached to the skin near selected blood vessels. Our future clinical goal is to detect CTCs in hand vessels with diameters of 1–2 mm at 1–3 mm depths (easily accessible with PAFC) in which 3–5 L of blood circulates approximately during one hour. In preclinical testing on the mouse model this device could detect up to 72% of pigmented human melanoma CTCs and approximately 20–40% of low pigmented cells. These data obtained by comparison of PA signals from nonlabeled and labeled by NPs melanoma cells can be used for estimation of the false negative rate and hence the correction of experimental data.

We believe that the PAFC technology may have a tremendous clinical significance due to its high sensitivity and lack of a time-consuming labeling procedure. It can indicate the presence of CTCs in the blood at an extremely low concentration, much below the sensitivity threshold of other methods. Clinical applications may include: (1) blood screening for early CTCs before metastases progression; (2) testing for cancer recurrence; (3) individualized assessment of the therapeutic intervention (e.g., surgery, chemo, or radiation) and its efficiency through real-time CTC counting; and (4) potential for metastasis inhibition, if not prevention by a well-timed therapy. We identified the ways for further PAFC's improvement: (1) decrease the background signals from blood through changes in its oxygenation, osmolarity, and hematocrit, within physiological norms [30]; (2) assessment of CTCs in deep large vessels (e.g., jugular vein) with a focused ultrasound transducer [30]; (3) increase PA contrast by drug-induced activation of melanin synthesis in melanoma cells [30,56]; (4) melanogenesis activation in melanoma and even non-melanoma (e.g., breast cancer) cells via transfection with tyrosinase-activating plasmids [30,56]; (5) use melanin nanoparticles as new PA contrast agents [37] for targeting of melanoma and other nonpigmented CTCs; and (6) targeting of melanoma CTC *in vivo* by magnetic NPs conjugated with specific antibody and magnetic CTC enrichment [37].

3.2. Real-time monitoring of circulating tumor cells released during intervention

For many years, oncologists believed that some medical intervention may provoke metastasis; however, no direct evidences were previously presented. Using label-free PAFC and the melanoma-bearing mouse model (Figs. 1A and 3A), we discovered that palpation, biopsy, conventional and laser surgery may either initiate CTC release in the blood which previously did not contain CTCs, or dramatically increase (10–30-fold) the CTC counts

above the previous level, which can increase the risk of metastasis. In particular, the 120 g weight pressure or palpation (by squeezing of the melanoma tumor with fingers), notably increased the CTC count (Fig. 1B) that eventually led to the appearance of lung metastasis at week 3 after tumor implantation, compared to no metastasis without the pressure of palpation. The damage of blood vessels in the tumor during biopsy or conventional surgery, modeled by a small, scalpel-induced incision or laser tumor treatment, led also to the appearance of CTCs in the circulation. On the contrary, complete tumor resection by cutting tissue around the localized tumor, led to the disappearance of CTCs in circulation within a few hours (Fig. 4A), suggesting the primary tumor as the main source of CTCs. However, in several cases, CTCs appeared again in the circulation a few weeks after surgery, which might indicate the influence of metastasis in the distant organs or cancer recurrence in the primary tumor site. Although the animal model was used in this study, our results may warn to oncologists of precaution during physical examination, take a careful surgery plan, or indicate the importance of adjuvant or preventive anti-CTC therapy during primary tumor treatment. Patients should not wear tight clothes to avoid skin pressure above the tumor.

Our data shows that PAFC can be used both as a research tool on animal models to provide insights on the potential of various therapies to provoke metastasis and as a clinical instrument for personalized cancer diagnosis, and for a guidance of appropriate therapy. In particular, when we exposed an abdominal vessel by an 820 nm- wavelength laser, increasing the energy fluence from 60 mJ/cm² to 600 J/cm² led to an increase of PA contrast of the CTCs above the RBC background by ~6 times [30]. This phenomenon was associated with laser-induced nanobubbles around overheated strongly absorbing melanin nanoclusters in melanoma cells, which served as a nonlinear PA signal amplifier compared to linear PA signals from RBCs with homogenous hemoglobin distribution (i.e., with no nanobubble formation). On the other hand, the rate of CTCs have been gradually decreased from 12 to 1–2 CTC/min over a 1 h monitoring period (Fig. 4B). This effect was also associated with the generation of nanobubbles as a melanoma cell killer [30,59]. Nevertheless, later the CTC rate gradually increased to almost levels initially detected, indicating the appearance of new CTCs from the primary tumor. These data demonstrate a great potential of PAFC for guiding blood purging *in vivo* by periodically exposing the blood vessels to the laser. Further study could determine whether this new treatment is effective enough to be used alone, or whether it should be used in combination with chemo or radiation therapy.

Using advanced PAFC to monitor CTCs in mice models with different melanoma tumors, we discovered the early appearance of CTCs during the first week of tumor development, but the CTC rate fluctuated and completely disappeared at the late stage of tumor development in 30–40% of the cases, thus demonstrating a low correlation of CTC counts with tumor size. This phenomenon can be related either to the high heterogeneity of local tumor environments, a poor blood network in large tumors, or the development of less metastatically-active cells both in the primary tumor and in distant metastasis. This important observation requires further study with a specific focus on the role of CTC count as a marker of tumor progression and metastasis development. *In vivo* flow cytometry could be the key tool to address this important issue.

3.3. Multiplex molecular targeting and magnetic enrichment of breast CTCs

A combination of conventional flow cytometry with separation, isolation and enrichment methods revolutionized diagnosis and therapy of diseases [60]. Various physical cell properties, including size, motility, electrical dipole moments, as well as optical and magnetic qualities have been exploited for this purpose. In particular, specific cells (e.g., CTC or bacteria) or biomolecules (e.g., proteins, and DNA) in biological fluids such as blood, urine, or cerebrospinal liquids, were labeled using magnetic microbeads and MNPs and then they are separated and enriched from the sample flow by a magnetic field [60]. To date, these techniques are used only in *ex vivo*, while the applications *in vivo* are limited by static objects only. We demonstrated for the first time, the application of magnetic enrichment of CTCs directly in bloodstream (Fig. 5A–D) [28].

In light of the limited expression of most cancer markers, we applied a multiplex targeting strategy for the detection of CTCs. Specifically, we used duplex molecular targeting of the MDA-MB-231 human breast cancer cells, which is positive for urokinase protease-activated receptors (uPAR) and folate receptors (FRs). It has been demonstrated that 60–90% of breast cancers express uPAR ($\sim 10^5$ receptors/cell vs. 2,500 receptors/cells for normal human epithelial cells) [28] and 80–90% express FRs [51]. Advanced golden carbon nanotubes (GNTs) (Fig. 5C) having an absorption maximum at 900 nm and a minimum at 639 nm (Fig. 5D) were coated with polyethylene glycol (PEG) and conjugated with folate. As the second NPs, 10-nm MNPs coated with PEG and amphiphilic triblock polymers were conjugated with the amino-terminal fragment (ATF) of a urokinase plasminogen activator (uPA), which is a high-affinity, natural ligand for uPAR (Fig. 5B). MNPs have a absorption in the broad NIR range; nevertheless, the absorption spectrum is different from that of GNTs (Fig. 5D). PA and PT scanning cytometry [22,27,30], in combination with fluorescent imaging, revealed that this NP cocktail exhibited the best targeting efficiency (96%) *in vitro*, in a blood sample spiked with rare tumor cells and a negligible level ($\sim 6\%$) of background PA signals from unbound or nonspecifically bound NPs. At weeks 2, 3 and 4 of tumor development in mouse models (Fig. 6A), a cocktail of the conjugated NPs were injected intravenously into the circulation. PA monitoring of targeted CTCs at 20 min after injection (to allow clearance of most unbound NPs) showed that the ratio of the numbers of CTCs in the ear of the mouse compared to those in abdominal vessels (CTCs/min) increased from $(0.9 \pm 0.3)/(6 \pm 2.1)$ at week 2 to $(7.2 \pm 0.3)/(26 \pm 2.1)$ at week 3 and to $(15.1 \pm 2.7)/(47 \pm 6.4)$ at week 4 (Fig. 6B). These data approximately correlated with the stage of the tumor progression. Attaching a magnet with a field strength of 0.39 Tesla led to immediate increases in both PA signal amplitude and rate, and changed the character of the PA signal from infrequent flashes to a continuous increase of permanent PA signals up to 88-fold within 1.5 h, indicating successful magnetic CTC capturing [28]. Applying this method to clinical use, the patients may potentially carry a magnet attached to selected peripheral vessels (e.g., in wrist area) for trapping of CTCs, followed by a quick PA detection of the trapped CTCs, and, if necessary, local PT treatment or removal of CTCs by using syringe-based systems for further molecular analysis.

3.4. Flow cytometry platform for detection and killing of circulating cancer stem cells

It is believed that a small population of cancer cells (3–5%), called tumor-initiating or cancer stem cells, may be a cornerstone of metastatic initiation and progression due to their extensive self-renewal capacity, tumorigenicity and multipotentiality associated with drug and irradiation resistance [61]. Thus, these cells could be a novel and crucial target for diagnosis and therapy. However, little is known about subpopulations of these cells that can enter the circulation and migrate to distant sites, forming metastases. Because an extremely low concentration of stem CTCs are expected in the circulation, we proposed to use high sensitivity PAFC for detection of stem CTCs [31]. Specifically, *in vivo* targeting of breast CTCs with a stem-like phenotype, which are naturally shed from the parent tumor in mouse models, were performed with functionalized gold-based GNTs and MNPs. Data *in vivo* were verified *in vitro* using PA and PT scanning cytometry. We discovered that magnetic-induced clustering of MNPs in individual cancer cells (MDA-MB-231) led to significant (10-fold) amplification of PT and PA signals. We also demonstrated the proof-of-concept *in vitro* that PA diagnosis can be integrated with targeted PT eradication of individual stem CTCs.

In the preliminary study *in vivo*, GNTs and MNPs conjugated with folic acid and antibodies to CD44 were selected for the detection of stem-like CTCs. For identification of bulk CTCs, we used markers described in Section 3.3. At week 4 of the tumor inoculation, when the metastatic disease was well recognized by metastasis in the distant organs (e.g., liver), MNPs-Folate and GNT-CD44 were separately injected into a vein in the mouse tail. To allow effective labeling of CTCs in the bloodstream and washing out of unbound NPs, PA monitoring of blood vessels began 20 min after injection. As expected, the flashing readable PA signals above the PA background of blood were detected within 2 h of the observation. This indicated approximately 10–15% of stem-like CTCs were among the bulk CTCs (Fig. 6C). To the best of our knowledge, we demonstrated for the first time, that the multifunctional PAFC-PT-nanotechnology-based-platform has the potential for ultrasensitive PA molecular detection of stem CTCs *in vivo*. The interpretation of the obtained data at the current stage requires further studies, which are now in progress in our laboratory. It includes exploring the role of the folate receptor and adding CD24 and CD45 markers to increase detection and specificity of stem-like CTCs and excludes possible false positive signals from leukocytes, respectively.

3.5. Detection of disseminated tumor cells in lymph flow and sentinel lymph nodes

The fact that common pathways for the dissemination of cancer cells from the primary tumor are lymph and blood vessels, and that tumor cells may pass from one system to another through numerous anatomical interconnections between lymph vessels, sentinel lymph nodes (SLNs) and the blood circulatory system has been well-known for many years (see [62] and references there). Nevertheless, until now, so-called disseminated tumor cells (DTCs) and CTCs in lymph and blood systems, respectively, have been studied separately. Moreover, most efforts have been focused on the examination of CTCs in the blood system, while studies of the metastasis process in lymphatics were paid much less attention to.

Compared with the blood vasculature, lymph vessels are colorless, with a relatively low pressure and low concentrations of cells. As a result, lymph sampling is impractical due to

its yields of only a few microliters at a time and long-term cannulation. Recently, we integrated *in vivo* blood and lymph PAFC, PA lymphography, and PA scanning cytometry (study of nonmoving cells), and demonstrated a potential of this platform for real-time, *in vivo* quantitative monitoring of CTCs in blood, DTCs in prenodal lymphatics, and SLNs, using intrinsic melanin or functionalized NPs as PA contrast agents in melanoma and breast tumor bearing mice models, respectively. It allowed us to define cross-correlations between lymph DTCs, blood CTCs, the size of the primary tumor, and nodal and distant metastases (Table 1). Specifically, in the preclinical mouse melanoma model, we revealed that early metastatic cells in latent metastatic disease (4–7 days after tumor inoculation) are equally disseminated through blood and lymph pathways. However, in a few cases, metastatic cells appeared in lymph vessels at week 1 without any cells detected in the blood vessels and *vice versa*, suggesting an individualized character of tumor cell dissemination. During week 2, a 3.5-fold primary tumor growth was accompanied by a 10-fold increase of DTC count in the lymph flow and by a 6.5-fold increase in the number of PA signals in SLNs as a sign of metastasis development (Fig. 7), while much less CTCs were observed in the blood flow. PAFC demonstrated an unprecedented sensitivity threshold for *in vivo* lymph testing, as one melanoma CTC in the background of 10^6 WBCs could be identified [20]. The association between DTC count and SLN metastasis progression supports lymphatic DTCs as a novel prognostic marker of metastasis.

In addition, we demonstrated PAFC-guided, PT purging of melanoma and breast cancer micrometastasis in SLNs mimicked by direct injection of cancer cells in SLNs. Targeted detection of breast cancer metastasis in the SLNs of the mouse models was performed by injection of the GNT-folate conjugates in the mouse ear. At five minutes after the injection, strong PA signals above the background, with a contrast of 10–15 appeared in the SLN due to the transportation of the NPs through the lymph vessels to the SLN and the targeting micrometastasis in the SLNs. Subsequent application of the therapeutic laser pulses with enhanced energy fluence led to a decrease of these initially PA strong signals to a background level, which indicated laser-induced destruction of tumor cells. On the contrary, when the experiment was repeated with unconjugated GNTs, low PA signals with a contrast of ~3 above the background were observed, suggesting random distribution of GNTs in the SLN volume. To verify these data, *ex vivo* experiments mimicking the lymph node micrometastasis were carried out using conjugated GNT-folate and fluorescent dye for visualization of the tumor cells [22]. These results support the feasibility of the theranostic PAFC-PT platform for the *in vivo* detection and killing of metastatic cells in SLNs exploring NP clustering in micrometastasis accompanied by red-shift effect and PA signal amplitude enhancement as an indicator of molecular targeting [59]. This platform, in combination with microarrays, might provide assessments of the biological properties of lymph DTCs (e.g., molecular profiles and viability) compared to those in the primary tumor, regional and distant metastasis, and blood CTCs during metastasis progression, with a focus on identifying the tumor initiating cells among the bulk lymph DTCs. The expanded knowledge of lymphatic-related metastasis may catalyze a paradigm shift in the diagnostic clinical oncology, from conventional assessment of early metastasis in SLNs toward lymph DTC testing. Taking into account the safe nature of the proposed *in vivo* lymph cancer tests as

supplementary (or in some cases as alternative) to conventional blood tests, we anticipate a quick translation of this technology for use in humans.

3.6. In vivo photoacoustic detection of circulating tumor cells in cerebrospinal fluid

The dissemination of cancer (e.g., leukemia, lymphoma, breast cancer, and melanoma) into the central nervous system either through hematogenous spread, direct release from the tumor itself, or by migration along perineural or perivascular spaces is a serious medical problem leading to neurological symptoms (e.g., neoplastic meningitis) and rapid mortality [63]. In particular, the presence of tumor cells in cerebrospinal fluid (CSF) may serve as a marker of disease progression and early-stage brain metastasis in breast cancer. The CSF is a colorless body fluid with a total volume of 135–150 mL in adults, which circulates through the ventricular system around and inside the brain and the spinal cord. The CSF flow has pulsatile “forward-backward” characteristics that correlate with cardiac cycles. Forward velocity in the spinal canal ranges from 10 mm/s in the craniocervical junction to 1 mm/s in the lumbar part of the canal; the velocity of backward flow is approximately two times slower than that of forward flow. The CSF function is bathing the central nervous system, and bridging the vascular and lymphatic systems. The current tools available for detecting CTC spread into the CSF such as cytology, *in vitro* flow cytometry and others [63,64] are limited by small sample volume and suffer from a lack of sensitivity, leading to delays in treatment. Improved detection of CSF malignancy is a clinical imperative.

We propose that many of these problems could be resolved by increasing the sensitivity and specificity of CSF examination through *in vivo* analysis of a relatively large volume of circulating CSF using noninvasive PAFC, and functionalized NPs as PA contrast agents. This approach has not been attempted previously. In our study, gold nanorods (GNRs)-folate conjugates with maximum absorption near 670 nm were injected in primary tumor of tumor-bearing mice 10 weeks after tumor inoculation. The next day irradiation through a 300- μ m fiber of CSF in the cisterna magna of nude mice with a nanosecond laser pulse at 671 nm revealed rare PA signals with a rate of a few signals per hour associated with targeted breast tumor cells flowing through the CSF. Using the methodology described in Sections 3.3–3.5, the assessment of the brain confirmed the presence of micrometastasis as a potential source of tumor cells in the CSF. The estimated sensitivity was approximately a few CTCs among a million leukocytes over a four-hour period. Our approach may provide a semi-automated PA molecular analysis that vastly improves the sensitivity, reliability, objectivity, and accuracy of detecting tumor cells in CSF compared to CSF cytology. Due to high depth assessment (up to few cm), fiber-based PAFC could be applied to different sites of the spinal canal (e.g., cervical, lumbar) and ventricles. If clinically successful, this tool may provide a tremendous leap forward in a previously unexplored scientific area of *in vivo* CSF testing including cell trafficking and counting, cell-to-cell interactions in the CSF, and the route of CSF circulation, especially in the terminal end of the spinal canal.

4. Detection and killing of circulating bacterial cells

Despite major advances in medicine over the last decade, microbiologically-based diseases continue to present enormous global health problems, especially due to the appearance of multidrug-resistant bacteria strains. The critical steps in the development of bacterial

infections include their penetration into the blood system, interactions with blood cells flowing in the circulatory system, or with endothelial cells, and further translocations in the host organisms. Unfortunately, little is known about circulating bacterial cell (CBC) kinetics in the blood pool. This includes their clearance and adherence rates, which might be very important for understanding the transition from the bacteremic stage to the tissue invasive stage and development of an effective therapy. Previously, we demonstrated the capability of the PAFC-PT platform for *in vitro* detection and killing of *S. aureus* and *E. coli* labeled with gold NPs and carbon nanotubes (CNTs) [15,65,66]. Recently, we extended this platform for *in vivo* magnetic enrichment, multiplex PA detection and PT eradication of circulating *S. aureus* (Fig. 8A) using the methodology developed for CTCs [28]. Bacteria were targeted directly in the bloodstream through intravenous injection of silica-coated MNPs (siMNP) and GNRs (with a maximum absorption near 820 nm) functionalized with antibodies specific for *S. aureus* surface protein A and lipoprotein, respectively. These are both highly expressed in *S. aureus* and absent in mammalian cells. After successful two-color PA detection of targeted CBCs at low energy fluence (50 mJ/cm²) of lasers at 671 and 820 nm, mice were subjects of PT therapy by a one-hour laser exposure of a 300- μ m abdominal blood vessel with a laser fluence of 800 mJ/cm² at 820 nm, coinciding with an absorption spectra of GNRs. PT nanotherapy led to a significant decrease in the PA signal rate (Fig. 8B, red curve) compared to the control group (blue curve) at a laser fluence shown to be safe for blood cells. To confirm this therapeutic effect, mice were euthanized, and the blood was examined for the presence of viable bacteria. Blood from the control and PA-diagnostic groups showed similar bacterial growth, while the number of bacteria from the PT therapeutic group was reduced by 10-fold.

Compared to existing diagnostic and therapeutic approaches, the PAFC-PT nano-theranostic platform may offer many advantages: (1) ultra-high sensitivity (0.5 CFU/mL); (2) physical, PT-based destruction of bacteria, thus retaining its therapeutic efficacy irrespective of the antibiotic resistance status of the offending bacteria; (3) integration of multiplex PA molecular detection and PT targeted elimination of CBCs with real-time PA monitoring of therapeutic efficacy; and (4) high spectral specificity based on distinct spectral properties of NPs [41]. Because some gold NPs were already approved for pilot studies on humans [28], and the clinical potential and safety of PA technology has been successfully demonstrated in pilot trials [46–49], early laser-based detection and treatment of CBCs could be feasible in a 0.5–1.5 mm hand vein at 1–2 mm depths, which is within the well-documented capacity of the PA technology to assess much deeper (5–7 cm) and larger (1 cm) human blood vessels [46].

5. Detection of circulating clots using negative and positive photoacoustic contrasts

When a blood vessel is injured, the normal physiological response of the body is clot (thrombus) formation to prevent blood loss. Alternatively, even without vessel's injury, pathological condition called thromboembolism may lead to formation of circulating blood clots (CBCs) that eventually can plug the vessels of different locations, in particular, in the veins of extremities (venous thromboembolism), lungs (pulmonary embolism), brain

(embolic stroke), heart (myocardial infarction), kidney, or gastrointestinal tract. Thromboembolism is a significant cause of morbidity and mortality, especially in adults. Despite clear medical significance of CBCs little progress has been made in the development of methods for real-time detection and identification of CBCs. Many CBCs remain undetectable, unless they result in clinical phenomena. The majority of patients die because of a failure in diagnosis rather than inadequate therapy. Commonly used *ex vivo* methods of detecting clots are time-consuming, low sensitive due to small volume blood samples, and are limited by discrete time-point sampling with difficult access to clinically relevant sites. Most *in vivo* methods are only able to detect fixed or slowly moving large clots. A pulse Doppler ultrasound is a promising technique for the detection of CBCs, but this technique cannot assess clot composition, detect small clots, and may be affected by artifacts [36 and refs. there].

To overcome these limitations, we proposed an *in vivo* PAFC for real-time detection of clots of different compositions (Fig. 2A) [36] using a combination of positive and negative contrast modes. Laser irradiation of blood vessels in normal vessels creates a constant PA background determined by the absorption of RBCs randomly distributed in the irradiated volume. Depending on the size of the vessels, hematocrit (Ht), and PAFC spatial resolution, the number of RBCs in the detected volume can vary from one or a few RBCs in the capillary to thousands in larger vessels. When a RBC-rich red clot passes through the irradiated blood volume, a transient increase in the local absorption which is associated with a high concentration of hemoglobin (Hb), results in a sharp positive PA peak (Fig. 2C, left). Red clots can be detected when they have a higher local absorption than the normal RBC background in the detected volume. For example, five RBCs in a red clot have a volume of ~450 fL (corpuscular volume of a single RBC is 90 fL [35]) with a total Hb amount of 150 pg (~30 pg of Hb per one RBC), while the same volume of whole blood with a Ht of 45% contains just two RBCs with 60 pg of Hb. Thus, the expected ratio of PA signals from even a small RBC number in the clot to blood background is 2.5, which is sufficient for the detection of RBC aggregates. When a weakly-absorbing, platelet rich white clot (Fig. 2A) passes through the laser-irradiated vessel volume, a transient decrease in the local absorption results in a sharp negative PA signal (Fig. 2C, middle). A mixed clot with both RBC-rich (i.e., high-absorbing) and platelet-rich (i.e., low-absorbing) local zones will produce a pattern of positive and negative PA signals (Fig. 2C, right).

This phenomenological model was first verified *in vitro* using human blood, then *in vivo* in animal models by injection of well-established agents for the creation of circulating clots, and eventually in preclinical studies using the mouse model of myocardial infarction [36]. The readable transient PA signals were observed with different patterns of negative, positive, and combined contrasts (Fig. 9A) associated with white, red, and mixed clots, respectively, compared to no signals in control normal mice. The concentration and size of clots were measured with a threshold of a few clots in the entire circulation (i.e., 1–3 clots/mL in mice) with the size as low as 20 μm that is unachievable with the existing techniques. This PAFC-based diagnostic platform can be used in real-time defining risk factors for cardiovascular diseases, as well as for the prognosis and potential prevention of stroke by using a well-timed therapy or for a clot counting as a marker of therapy efficacy.

It is believed that clots are also common complications of infection, inflammation, cancer, surgery, radiation and coronary artery bypass graft (CABG resulting in heart attacks and strokes (e.g., [67,68])). However, due to restrictions of existing assays, little data has been available of CBC detection during these complications. Fig. 9B illustrates PA detection of clots during melanoma development on the melanoma-bearing mouse model. PAFC allowed to identify clots traveling separately or together with melanoma cells through temporal coincidence of CTC-related positive peaks and negative peaks associated with white clots, respectively. PA data supports the hypothesis of platelets aggregating around CTCs. We also observed that a surgery modeled by surgical removal of tissue or local incision led to the immediate appearance of circulating white clots associated likely with aggregated platelets (Fig. 9C). Thus, the PAFC-based diagnostic platform can provide insights on clot formation occurring in different diseases, pathological states or therapeutic interventions. This platform has a potential for real-time defining of risk factors for cardiovascular diseases, for prognosis and potentially prevention of stroke by well-timed therapy, and estimation of therapy efficacy through PA clot counting.

6. *In vivo* fluctuation flow cytometry for dynamic monitoring of red blood cell aggregation and sickle cells

Alterations in blood rheology (hemorheology) are important for the early diagnosis, prognosis, and prevention of many diseases including sickle cell anemia, thromboembolism, trauma, inflammation, diabetes, heart attack, stroke and malignancies [35]. However, real-time *in vivo* assessment of multiple hemorheological parameters over long periods of time has not been developed. PAFC alone, or in combination with PT techniques has the capability of label-free, dynamic monitoring of hemorheological parameters *in vivo* including RBC aggregation, shape and deformability, hemoglobin distribution, individual cell velocity, shear rate and viscosity [35]. The detection of RBC aggregation and sickle cells can be performed by so-called *in vivo* fluctuation PAFC. Irradiation of blood vessels created a different PA background pattern determined by vessel diameter and RBC properties (Fig. 2A). The average level of this background is proportional to the number of RBCs in the irradiated volume, thus allowing the estimation of the Ht after the corresponding calibration procedures taking into account blood oxygenation, Hb concentration, and vessel size [35]. The fluctuation in the background absorption is associated with changes in the number of RBCs in the irradiated volume. This fluctuation increases as the vascular diameter decreases. Ultimately, in a capillary with single-file RBC flow, single flash PA signals appear when individual RBCs sequentially pass through the irradiated volume (Fig. 2D, left trace). In arterioles and venules in which several RBCs appear simultaneously in the irradiated volume, the background level increases, but its fluctuation decreases. In large vessels containing hundreds or thousands of RBCs in the irradiated volume, the background continues to rise and fluctuations are minimized and are ultimately determined by laser pulse energy instability. The dilution of blood with a physiological solution leads to a decrease in the background level that can be used to calculate Ht [35].

When many small RBC aggregates or a single large RBC aggregate passes through the irradiated volume, it results in an increase in PA signal fluctuations (Fig. 2D, third trace) and/or transient strong PA peaks (Fig. 2D, fourth trace), respectively. The increase of local absorption in two- and three-dimensional aggregates leads to a significant increase in PA signal fluctuations as a marker of either normal formation of reversible (physiological) rouleaux or irreversible (pathological) clumps of RBCs. These data were obtained in the mouse model by intravenous injection of Dextran500 (a well known agent for activation RBC aggregation) [35]. In 40 μm ear blood vessels a few minutes after injection the PA signal amplitude fluctuations increased on 10–30% as compared to control blood vessels, and occasionally large PA signal peaks were observed (Fig. 2D, fourth trace). These data suggest that PA signal fluctuations and PA spectral analysis can be used to monitor RBC aggregation. In particular, analogous to large CNT clusters [33] and clots [36], we observed that the shape of individual PA signals from relatively large RBC aggregates is changed and accompanied by a shift of the central frequency in individual PA signal spectra to that of a lower frequency. Therefore, analysis of spectra of individual PA signals (Fig. 2A, top right) as well as the amplitude and width of averaged peaks in PA signal traces (Fig. 2C) can provide information of the average aggregate size, and potentially its two- and three-dimensional spatial configuration.

A similar approach can be used to study sickle cell disease (or sickle-cell anemia) associated with a genetic modification of RBCs which result in the production of abnormal hemoglobin, HbS instead of hemoglobin HbA. This point mutation exerts its effects by causing precipitation and polymerization of the deoxygenated HbS, resulting in the sickling of RBCs. The elongated-shaped, rigid, sickle cells lack deformability, occlude the microvasculature, and lead to tissue infarctions, which manifest as a painful sickle cell crises. Optical and other methods have helped to develop an explanation for the sickle crises caused by cell adhesion to the vascular endothelium followed by “log-jamming” of the rigid sickle cells, which has stimulated much research into new treatments. However, characterization of sickle cell properties at the single cell level has been poorly explored. We believe that fluctuation PAFC integrated with PT techniques could benefit sickle cell disease research by focusing on *in vivo* study of the behavior of sickle cells in a real biological environment.

Using a genetically modified mouse model of human sickle cell disease, high-sensitivity PT imaging demonstrated the ability to distinguish sickle RBCs on the basis of their specific shape and intracellular PT patterns [35]. Sickle RBCs had a more profound spatial heterogeneity than normal RBCs which may be associated with increased clustering of HbS compared to HbA. Comparison of PA, PT and the conventional absorption spectra of normal and sickle RBCs demonstrated a slight difference in the spectral range of 590–660 nm which makes spectral identification difficult.

However, both PT and PA signal amplitudes from normal RBCs were 2–7-fold higher than the amplitudes from sickle RBCs. This difference in amplitudes was associated with the specific cell shape and Hb concentration (Fig. 10). As a result, the assessment with PAFC of similar size the ear blood microvessels in normal and genetically modified mice revealed lower PA blood background and more profound PA signal amplitude fluctuations in mice

with sickle cells as compared to normal mice [35]. The level of these differences depended on the number of abnormal cells in circulation. Thus, *in vivo* fluctuation PAFC has the capability for real-time monitoring of RBC aggregation, sickle disease status and potentially the response to the corresponding therapy.

7. *In vivo* photoacoustic bone flow cytometry

We determined that even after significant attention of laser radiation in the bone tissue, the laser energy is still enough to generate readable PA signals from strongly absorbing objects inside bones using focused ultrasound transducers (Fig. 11A). Indeed, intravenous injection of carbon nanotubes (CNTs) led to the appearance of PA signal traces from the mouse tibia [39]. To exclude possible PA background signals from CNTs circulating in blood vessels between fiber used for delivery laser radiation and bones, the fiber was gently attached to the skin in an area with no visible vessels. A time-resolved detection system was used to select PA signals coming with a time delay from relatively deep bone compared to the depth of superficial blood vessels. Scanning of the laser beam along the bone revealed rare stationary PA signals associated with the accumulated CNTs in bone. Similar results were obtained for breast cancer cells (MDA-MD-321-GFP) targeted by GNRs with a maximum absorption at 670 nm. The GNRs conjugated with folate were intravenously injected in the tumor-bearing mouse model at week 4 after tumor inoculation. We observed rare PA signals from the tibia irradiated using a high-pulse-rate laser (10 kHz) at 671 nm (Fig. 11B). These signals were associated with individually targeted CTCs. PA scanning cytometry revealed also rare stationary PA signals associated with CTCs captured in bone. This technique, after further optimization, has the potential for early and painless diagnosis of bone cancer metastasis (noninvasive bone marrow biopsy) or infection through the administration of strongly absorbing NPs functionalized to identify specific cancer cells or infection markers. The identification of targeted cells can be performed in analogy to detection of micrometastasis in SLNs (Section 3.5). In particular, healthy bones produce low-level background PA signals because low concentrations of NPs are randomly and nonspecifically distributed in the bone tissue as compared to a high local concentration of NPs that is found in cells causing bone metastasis with possible red-shift effect in clustered plasmonic NPs [59].

8. *In vivo* photoacoustic plant flow cytometry

Understanding the nature of interactions between engineered nanomaterials and plants is very important to comprehend the impact of nanotechnology on the environment and agriculture. We demonstrated that an advanced imaging platform integrating genetic, PT and PA scanning methods can provide the study of NP-plant interactions in stationary condition at the single NP levels [69]. We developed also PAFC for the real-time noninvasive monitoring of nanomaterial transport in xylem and phloem of plant vascular systems [34]. The PA signals were detected in the plant using fiber to deliver laser radiation at 1064 nm (with minimum background absorption in plant tissue) and an ultrasound transducer attached to the plant surface. In particular, PAFC was able to monitor quantum dot-carbon nanotube (QD-CNT) conjugate uptake by the roots of the plants and trace its spreading through stem to leaves in a tomato plant. PA signals in the leaf mid-vein (Fig. 12A) were observed at ~5

min after the QD–CNTs were added to the water surrounding the roots (Fig. 12B). Taking into account the average distance between the roots and the detection point (5 cm), and some delay in NP uptake, the average linear flow velocity in the stem can be estimated as 0.2 mm/s. As xylem bundles are arranged symmetrically around the stem center, a fiber tip can easily be positioned against any part of the stem. In the stem, which was thicker than the leaf, laser radiation at 1064 nm was more strongly absorbed by deep stem structures providing an increase in the PA background (3–4-fold). Nevertheless, PAFC allowed the assessment of the QD–CNT aggregates passing through the stem [34]. We believe that PAFC using both endogenous and exogenous contrast agents have a potential to open new avenues of *in vivo* study of the transport the nutrients, products of photosynthesis and metabolism, NPs, infectious agents, and other objects through the plant vasculature.

9. Photoacoustic time-of-flight velocity measurement of single cells and nanoparticles

Existing optical techniques for *in vivo* measurement of blood flow velocity are not quite applicable for determination of the velocity of individual cells or NPs. PAFC may solve this problem by using time-of-flight technique with single or multiple laser beams (Fig. 13A). In the one-beam scheme, an object crossing a laser beam created transient PA signals with a width t_L , defined as $t_L \approx (D + d)/V_F$ where D is the diameter of object, d is the width of the linear laser beam, and V_F is the velocity of the object. Thus, V_F can be measured for objects and laser beam of known sizes. In the three-beam scheme, velocity of an object is determined by the time it takes for the object to travel between multiple laser beams (Fig. 13A, bottom). In this case, an object in flow produces multiple consequent trains of PA signals. Object velocity can be estimated by using the time intervals between the trains and the distances between the beams. This measurement is independent on the object size. These approaches were demonstrated *in vivo* using an animal (mouse) model by estimating velocity of GNRs, melanin NPs, RBCs, WBCs, and CTCs in the broad range of flow velocity from 0.1 mm/s to 20 cm/s [40]. In particular, monitoring of PA signal widths in mouse ear vessels from WBCs labeled with GNRs revealed the double maxima in peak-width histograms (Fig. 13B) associated with the fast moving portion of WBCs in central flow and slowly rolling WBCs. In general, the peak-width analysis in the time-of-flight mode provided important information on objects' velocities, cell aggregation manifested by the appearance of wider peaks with complex shapes, and on rolling effects that result in slower cell velocity. However, width histograms alone were not sufficient to distinguish a large aggregate of cells with high velocity and an individual rolling cell moving slowly because both would produce peaks of similar widths. We determined that these objects could be identified with a two-parameter plot representing PA peak amplitude and peak widths obtained from time-of-flight measurements. Fig. 13C illustrates verification of this approach for label-free detection of melanoma B16F10 cells. Three areas of interest were selected for this scatter plot: I) the area with the highest density of events, corresponding to fast-moving individual cells; II) aggregates of cells that were larger and had more melanin than individual cells; and III) peaks with PA signal too small to consider these objects as aggregates. Large peak widths in III suggested that these cells were moving more slowly

than the other cells. Therefore, multiparameter analysis of PA data may provide a criterion for distinguishing aggregates and individual rolling cells [40].

10. Pharmacokinetics of circulating contrast agents: NPs, dyes, liposomes, and microbubbles

The rapidly growing application of *in vivo* imaging modalities (e.g., optical, MRI, or ultrasound) and nanotechnology-based probes has placed new demands on monitoring the clearance rate of various contrast agents including NPs, drug carriers (e.g., liposomes), dyes and microbubbles. No clinically relevant method has been developed for rapid and ultrasensitive study of the pharmacokinetics of these agents including their clearance rate in different vessels. As most contrast agents have intrinsic optical absorption, PAFC is an almost ideal tool for real-time, label-free monitoring of their pharmacokinetics. We demonstrated PAFC's capability to monitor the circulation lifetime of NPs (e.g., GNRs, GNTs, MNPs), dyes (ICG, Methylene Blue [MB], and Trypan Blue [TB], Evans Blue, and Lymphazurine), microbubbles, normal blood cells, and CTCs (melanoma, breast cancer, squamous carcinoma, pancreatic cancer) using a laser with different wavelengths [11,15,28–30,33,38,40]. Circulating objects can be detected when PA signals from them are above the blood background associated preferentially with absorption of RBCs (Fig. 14A, top). After injection of contrast agents, PAFC provides two typical signal trace patterns (Fig. 14 middle and bottom) as an increase in the baseline level above blood backgrounds and strong fluctuations above the baseline. The first pattern is associated with homogenous random distribution of dye molecules, individual microbubbles, or NPs in the detection volume, while the second pattern is related with the presence of their aggregates with a more strong localized absorption. The clearance rate for most NPs depending on their protective layer properties, was in the range of 20–40 min, and in rare cases a few hours, while liposomes demonstrated a long term circulation of up to few days [42]. Some NPs demonstrated aggregation in blood flow especially during injection procedures. The described approach should be useful to the routine evaluation of the possible influence of the natural properties of NPs, the protective materials, and the coating procedures on NP clearing. The approach *in vivo* allows minimization of the animal number compared to *ex vivo* methods with periodical blood sampling.

Intravenous injection of normal blood cells and tumor cells labeled with various contrast agents and having different functional states revealed their different clearance rates (Fig. 14B): 1–3 min for necrotic cells, 5–15 min for apoptotic cells, 30–60 min for high metastatic tumor cells, one-three hours for cancer cells with lower metastatic activity, and several days for normal RBCs and WBCs, respectively. These data showed that the immune responses to abnormal and especially metastatic active cells were stronger than those to normal blood cells with labels.

PA monitoring of the clearance of dyes (ICG, MB, and TB) revealed increased dynamic PA signal fluctuations, which can be associated with interactions of dyes with circulating blood cells and plasma proteins [38]. The injection of TBs, which is broadly used for viability tests *in vitro*, led to rare notable PA signals *in vivo* which can be associated with dead cells uptaking TB directly in bloodstream. Thus, in addition to previously demonstrated detection

of circulating normal and apoptotic cells [20], we demonstrated the potential of PAFC for detection of circulating rare dead cells. This is important for many applications including studies of cell metabolism in normal and pathological states or response to various therapies. PAFC can provide also detection of circulating microbubbles labeled with NPs (e.g., GNTs) as an ultrasound and PT contrast agents for imaging and thrombolysis of clots. Later was based on laser-induced transient microbubbles using stationary microbubbles labeled with NPs [39]. The typical clearance rate of microbubbles was in the range of 5–10 min.

11. Ultrasharp nonlinear photoacoustic and photothermal resonances for multicolor flow cytometry

PAFC's selectivity to identify many disease-associated markers can be limited by the wide NIR spectral band (80–200 nm in width) of available contrast agents, in particular plasmonic NPs. Using the nonlinear laser-NP interaction, we demonstrated the “sharpening” of the broad absorption spectra of gold NPs to a 1–5 nm in PA and PT spectra [41]. Specifically, a tuning of the laser wavelength toward the absorption NP center leads to increased absorbed energy, raising the temperature above the nanobubble-formation threshold accompanied by significant (1–2 orders of magnitude) nonlinear PA/PT signal amplification. As a result, spectrally dependent signal amplification will lead to the sharpening of PA/PT resonances near the center of the absorption peaks at optimal laser energy providing nanobubble formation in this center only (Fig. 15A). At higher laser energy nonlinear spectral resonances can be red- or blue shifted from absorption peaks. For example, Fig. 15B demonstrates an asymmetric red-shifted resonance in GNRs with a minimum width of 0.8 nm limited by laser spectral line width. Similar nonlinear effects using pump–probe excitation results in the creation of ultrasharp dips [41]. This technique made it possible to easily identify each GNR in a mixture of seven NPs with nearly overlapping longitudinal plasmon resonances that were hardly distinguishable in conventional absorption spectra (Fig. 15C). Thus, up to 10–15 multicolor functionalized NPs whose nonlinear spectra do not overlap in the window of tissue transparency (650–1100 nm) can be used to simultaneously target 10–15 and potentially more biomarkers. It should be emphasized that tunable ultrasharp resonances can be created both in the center and outside the absorption peaks [41]. Indeed, at higher laser energy the threshold for nanobubble formation can be achieved at lower NP absorption. With even small laser wavelength shifts towards the absorption peaks, PA signal amplification is immediately changed on PA signal inhibition due to ultra-fast reaching of the threshold of NP destruction during laser pulse through thermal-based NP melting and explosion [41]. It is important to note that nonlinear, ultrasharp PA spectral resonances are accompanied by amplification of PA signals that lead to dramatic increases in both the specificity and sensitivity of PAFC.

12. Combination of photoacoustic and fluorescent flow cytometry

In vivo PAFC and fluorescence flow cytometry (FFC) systems were previously employed separately using preferentially positive contrast and a pulsed and continuous wave laser sources, respectively. Recently we introduced a multimodal *in vivo* flow cytometry (PAFFC, Section 2.3) which integrates both techniques, and can use positive and negative contrasts and pulsed lasers [42]. The supplementary nature of PA and fluorescence methods provided

dramatic increases in the detectable range of absorbing and fluorescent contrast agents. These included liposomes loaded with Alexa-660 dye, RBCs labeled with ICG, B16F10 melanoma cells co-expressing melanin and green fluorescent protein (GFP), C8161-GFP melanoma cells targeted by functionalized MNPs, MTLn3 adenocarcinoma cells expressing novel near-infrared iRFP protein, and QD–CNT conjugates. The use of a pulsed laser provided time-resolved discrimination of objects with a long fluorescence lifetime (e.g., QDs) from a shorter autofluorescence background (e.g., from blood plasma). Simultaneous PA and fluorescent detection of CTC–GFP (e.g., melanoma C8161-GFP) in tumor-bearing mice after injection of MNPs functionalized with antibody specific to melanoma receptors was useful for verification of CTC targeting by NPs directly in the bloodstream [42]. Our data showed that novel flow cytometry platform using negative contrast can provide label-free detection of low absorbing or weakly fluorescent cells in strong absorption blood background and autofluorescence background, respectively. Fig. 16 illustrates detection of low pigmented melanoma C8161-GFP cell which simultaneously provided positive fluorescence and negative PA signals. These data demonstrated that negative contrast label-free PAFC has the potential to detect single low absorbing cells with sizes down to 10–15 μm in 30–50 μm blood vessels (e.g., nonpigmented CTCs and likely WBCs). FFC also demonstrated the capability of label-free detection of red clots with weakly fluorescent RBCs providing negative contrast in blood plasma autofluorescence background, and white clots with fluorescent WBCs which provide positive contrasts above fluorescence background.

Thus, the combination of the positive and negative contrasts can be used for PA label-free enumeration of RBC and WBC aggregates, respectively. On the contrary, in FFC these cells and its aggregates produce label-free negative and positive contrast, respectively. This can provide synergy in identification of these cells using integrated PAFFC.

13. Conclusion

In this review, we have focused on the analysis of our recent results in the application of advanced *in vivo* flow cytometry using PA detection schematic and its combination with fluorescent techniques. The presented results suggest the excellent potential of PAFC as a new promising tool in biological research. This technology provides an unprecedented capability for real-time detection of tumor cells, bacteria, and clots in circulation with ultra-high sensitivity as one cells or clot in the background of billion normal blood cells. This is unachievable with existing techniques.

Analogous to conventional *in vitro* flow cytometry, *in vivo* PAFC may have a broad spectrum of similar applications *in vivo*, as well as provide many new applications that may include label-free detection of various objects with intrinsic absorption or targeted by functionalized PA probes (e.g., NPs) in a variety of vessels such as capillaries, veins, arteries and afferent and efferent lymphatics in assorted locations including ear, skin, and deep organs for many disease models (e.g., cancer, infections, cardiovascular or immune system disorders). Further development of this technology may solve very complex and largely unexplored areas of medicine related to detection *in vivo* of infectious agents and stem, dendritic, and metastatic cells in different functional states (e.g., apoptosis) in lymph and

blood flow at the single cell level. It may especially be used for early diagnosis of infections during its hematogenous spread with translocation of bacteria into different organs, vascular grafts, and stents. These infections commonly result in death caused by sepsis, despite aggressive treatment at the developed disease stage.

A transition of this technology to humans is anticipated with the development of portable devices attached to skin above selected blood vessels for alarm control of bacterial infection dissemination, cancer recurrence, metastasis development, and therapy assessment through controlling the number of circulating bacteria or metastatic cells, or monitoring of drug carriers (e.g., liposomes). One of the first clinical applications should be label-free detection of melanoma and circulating clots. The PAFC platform may allow the development of portable, personal flow cytometers for blood testing without a needle stick, using compact, robust, low-cost, laser diode arrays with different wavelengths. The ability to assess a large blood volume *in vivo* [potentially the patient's entire blood volume (in adults ~5 L)] may significantly (10^3 -fold) enhance the sensitivity of CTC detection including rare cancer stem cells compared to the existing CTC assay *ex vivo*. If oncoming pilot clinical trials using the portable PA flow cytometry device are successful, this technology can provide breakthroughs for the early detection of CTCs when metastasis has not yet developed and, hence well-timed therapy including PT therapy is more effective. The future developments of PAFC may include the use of integrated PA – Raman cytometry, or the identification of various hemoglobins (e.g., methHb, HbCo, and HbCN) and measurements of oxygenation at a single RBC level [70,71], and nano-theranostics as the integration of ultrasensitive PA diagnostics and multiplex nanotechnology-based targeted PT therapy.

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Fig. 1.

In vivo integrated PA and fluorescence flow cytometry. (A) Schematic for simultaneous detection of circulating cells (e.g., CTCs) with both absorption and fluorescence properties (right) during diagnostic and therapeutic interventions (left). (B) Example of a PA signal trace produced by melanoma CTCs (B16F10-GFP) in microvessels of the mouse ear before, during, and after pressure (120 g) applied on ~5 mm skin tumor. This figure is from a paper Mazen et al. [72].

Fig. 2.

The principles of fluctuation positive and negative contrast PA flow cytometry. (A) Schematic. (B) Absorption spectra of whole blood (red) and platelet-rich plasma (blue). (C) Example of PA positive, negative, and combined contrasts from circulating clots of different compositions. (D) PA signal trace dynamics obtained with PA fluctuation flow cytometry in different vessels in normal and pathological conditions leading to RBC aggregation.

Fig. 3.

In vivo label-free, PA detection of melanoma CTCs. Melanoma tumor growth in the mouse ear (A) and skin (B). (C) Average melanoma CTC rates in the ear and abdominal skin vessels, as well as the aorta, in B16F10 tumor-bearing nude mice 1 week after tumor development. (D) Change in the CTC count in the vessels of the abdominal skin as a function of time after B16F10 tumor cell inoculation in the ear (red empty circle) and skin (blue empty square). The dark red circle and blue square indicate averaged data. Laser parameters: wavelength: 904 nm; pulse energy fluence: 100 mJ/cm²; pulse rate: 10 kHz).

Fig. 4. CTC count as a marker of therapy efficacy. (A) Effect of incisional biopsy and complete resection on CTC dynamics. (B) PA guidance of PT therapy of CTCs.

Fig. 5.

In vivo magnetic enrichment and two-color PA detection of breast CTCs. (A) Schematic of setup. The laser beam is delivered either close to the external magnet or through a hole in the magnet using a fiber-based delivery system. (B) Schematic (left) and transmission electron microscopy image (right) of MNPs, each with a 10-nm core, a thin (2 nm) layer of amphiphilic triblock copolymers modified with short polyethylene glycol (PEG) chains and the amino-terminal fragment (ATF) of the urokinase plasminogen activator. Scale bar, 10 nm. (C) Schematic (left) and topographic atomic force microscopy image (right) of a GNT (12 × 98 nm) coated with PEG and folic acid. (D) PA spectra of 70- μ m veins in the mouse ear (open circles). Absorption spectra of magnetic nanoparticles (MNPs) and GNTs (dashed red and green curves) are normalized to PA signals from CTCs labeled with MNPs (filled red circle) and GNTs (filled green circle).

Fig. 6.

PA detection of bulk and stem-like breast CTCs in tumor-bearing mice. (A) The size of the primary breast cancer xenografts at different stages of tumor development. (B) The average rate of bulk CTCs in the mouse ear vein over a period of 1–4 weeks. (C) Average rate of CTCs associated with bulk Folate+/uPAR + CTCs and Folate-CD44 + stem-like CTCs in 200 μ m in abdominal skin blood vessels in the mouse model of breast tumor (at week 4).

Fig. 7.

PA scanning cytometry of melanoma metastasis in the sentinel lymph node (1.6×3 mm) at a single cancer cell level using the tumor-bearing mouse model at weeks one (left) and two (right) of tumor development. Red pseudo-color peaks indicate PA signals with maximum amplitudes. Each single spot is associated with single metastatic cells.

Fig. 8.

PA molecular diagnosis and photothermal-targeted eradication of circulating *S. aureus* in the blood of the mouse model with real-time PA monitoring of PT nanotherapeutic efficacy. (A) Schematic. (B) Experimental data. This figure is from a paper Galanzha et al. [73].

Fig. 9.

Label-free, real-time, PA detection of white, red, and mixed clots in the ear vessel of the mouse model in various disease states. (A) Monitoring of clots in the myocardial infarction animal model at a laser wavelength of 532 nm. The laser pulse repetition rate is 10 kHz. (B) Detection of clot formation in the blood vessels of the melanoma-bearing mouse model (at week 4). (C) Monitoring of clot formation during and after surgery modeled by small skin incision.

Fig. 10. Average PT signal amplitudes from normal and sickle RBCs of different shapes at a laser wavelength of 532 nm.

Fig. 11.
In vivo PA bone flow cytometry. (A) Schematics. (B) Noninvasive *in vivo* molecular targeted detection of breast CTCs in mouse tibia.

Fig. 12.

In vivo PA plant flow cytometry of QD-CNTs in tomato vasculature. (A) Linear laser spot in a leaf mid-vein, close to petiole. The red lines denote the part of the plant used for monitoring. (B) The trace of PA signals recorded in the leaf mid-vein after QD-CNTs were introduced into the water tank. Insets demonstrate the enlarged parts of the control trace and the trace fragment with signals from QD-CNTs. (C) The trace of PA signals from QD-CNTs recorded in the stem of the tomato plant. Black arrows indicate the moment QD-CNTs were introduced into the water tank. The PA traces before the introduction of QD-CNTs represent control data. Laser parameters: wavelength: 1064 nm; pulse width: 10 ns; pulse repetition rate: 10 kHz; laser pulse energy: 20 μ J; laser spot size in sample: 50 $\times$ 150 μ m (B) and 100 $\times$ 400 μ m (C).

Fig. 13.

In vivo PA time-of-flight velocity measurements of cells and nanoparticles. (A) Shapes of peaks in PA trace for particles of different sizes and various beam geometries. (B) Peak widths distributions in the WBCs in the mouse circulatory system targeted by GNRs with antibodies specific for the CD45 receptor. Laser parameters: wavelength: 820 nm, pulse width, 8 ns; pulse repetition rate: 10 kHz; laser pulse energy: 20 μ J. (C) Scatter plot of the height and width of peaks for label-free PA detection of circulating B16F10 cells in an arteriole of the mouse ear. Area I indicates signals from individual cells. Area II includes aggregates of several cells, and Area III indicates possible “rolling” cells.

Fig. 14.

PA monitoring of NP and cell clearance rates. (A) *In vivo* PA signal levels from the mouse ear vein and surrounding skin, compared to baseline noise, when the laser is off (top). Laser parameters: wavelength: 820 nm; fluence, 0.2 J/cm²; pulse rate: 10 kHz. PA monitoring of the clearance rate of 30-nm spherical gold NPs in a blood vessel of the mouse ear (middle). Laser parameters: 532 nm/cm², 9 kHz. PA monitoring of GNR clearance in a blood vessel of the mouse ear (bottom). Laser parameters: 1064 nm, 0.1 J/cm², 9 kHz. (B) PA monitoring of the clearance rates of melanoma cells at different functional states and metastatic activity and normal blood cells labeled with contrast dyes.

Fig. 15.

Ultrasharp nonlinear PA and PT spectral resonances. (A) Ultra-narrow resonances and dips in a homogenous absorption profile. (B) Asymmetric (~ 0.8 nm in width) resonance in GNRs. (C) Absorption and nonlinear PT spectra of a mixture of 30 nm gold nanospheres and six gold nanorods with spectrally different Plasmon resonances.

Fig. 16.

Positive and negative contrasts in PA and fluorescence flow cytometry. (A) Negative PA, and positive fluorescent contrast signals from circulating C8161-GFP cells in 40- μ m ear artery of the mouse. (B) *In vivo* fluorescent monitoring of white and red clots with positive and negative contrast, respectively. Laser excitation wavelength: 488 nm; intensity: 80 W/cm².

Table 1

Correlation between primary tumor size, metastasis in SLN and number of tumor cells in lymph- and blood flow.

	Tumor size (mm²)	Rate of lymph CTCs (cell/min)	Rate of blood CTCs (cell/min)	Number of PA signals associated with metastasis in SLNs	Histology
1 week	1.0 ± 0.2	0.26 ± 0.05	0.85 ± 0.03	493	No
2 weeks	3.6 ± 0.5	2.13 ± 0.30	1.07 ± 0.05	3,188	Yes