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In Vivo Flow Cytometry: A Horizon of Opportunities

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Abstract

Flow cytometry has been a fundamental tool of biological discovery for many years. Invasive extraction of cells from a living organism, however, may lead to changes in cell properties and prevents studying cells in their native environment. These problems can be overcome by use of *in vivo* flow cytometry which provides detection and imaging of circulating normal and abnormal cells directlyin blood or lymph flow. The goal of this mini-review is to provide a brief history, features and challenges of this new generation of flow cytometry methods and instruments. Spectrum of possibilities of *in vivo* flow cytometry in biological science (e.g., cell metabolism, immune function, or apoptosis) and medical fields (e.g., cancer, infection, cardiovascular disorder) including integrated photoacoustic-photothermal theranostics of circulating abnormal cells are discussed with focus on recent advances of this new platform.

Key terms

flow cytometry; spectral imaging; photoacoustic and photothermal methods; fluorescence; Raman spectroscopy; blood and lymph flows; circulating tumor cells; bacteria, and multicolor nanoparticles; theranostics

Introduction

Flow cytometry (FCM) is a well-established powerful analytical tool that has led to many revolutionary discoveries in cell biology and cellular-molecular disease diagnosis (1–5). In FCM, cells are introduced into a high speed (up to 5–20 m/s) laminar artificial flow, and after they are focused into single file, laser-induced fluorescence and/or forward and sideways scattered light from them 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. Recently an advanced Raman spectroscopy demonstrated the capability for high sensitive detection of individual particles in flow using Surface Plasmon Resonance Scattering (SERS) effects with signal integration times of 10 μsec (6–7). Many methods were developed for positioning cells and particles in a stream

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including mostly used hydrodynamic cell focusing (2) as well as dielectrophoretic, and acoustic focusing (8–13) and freely cell floating virtual-core flow cytometry (14).

Nevertheless, invasive extraction of cells from a living system may alter cell properties (e.g., morphology or marker expression) and prevents the long-term study of cell metabolism and cell-cell interactions (e.g., aggregation, rolling, or adhesion) in their native complex biological environment.

Other limitations of *ex vivo* FCM may include 1) low sensitivity for detection of rare abnormal cells (e.g., circulating tumor cells [CTCs]; 2) their time-consuming preparation procedures, taking many hours if not an entire day; and 3) the discontinuity of sampling with limited, discrete time points, making it difficult to detect early-stage disease and delaying effective, well-timed therapy. Indeed, in conventional FCM in the small volume of blood extracted (typically a few milliliters), no less than one abnormal cell (e.g., CTC or bacteria) can be detected. As a result, its current sensitivity level of 1–10 cells/ml is equivalent to 5,000–50,000 cells in the entire volume of blood (~5 L in adult), which is sufficient for the rapid development of a disease to a stage that is barely treatable or is incurable (i.e., metastasis, septic shock, or sickle cell crisis) (15–18). These shortcomings can be solved by development of a FCM instrument that allows *in vivo* noninvasive, continuous assessment of a significantly larger blood volume than current instruments and potentially a patient's entire blood volume (15). Detection and quantification of rare circulating cells *in vivo* is important for the early diagnosis of many diseases (e.g., cancer, infections, cardiovascular dysfunction, inflammation, immune disorders, or diabetes), or for the study of the influence of the different interventions (e.g., drugs, radiation, smoking, alcohol) on individual cells. In particular, to understand the mechanism of the development of metastasis, it is important to monitor tumor cell migration and interaction with other cells. And finally, *in vivo* study of apoptosis and especially circulating apoptotic cells with advanced molecular imaging is crucial for understanding human metabolic and immune system function.

FCM is the general term for quantitative detection, identification and enumeration of individual cells in flow (2,5,18). Most significant contribution in development of *in vivo* FCM for single cell analysis directly in the blood and lymph flow was made since 2004 by Vladimir Zharov's group in the University of Arkansas for Medical Sciences (15, 19–42) and Charles Lin's team in Wellman Center for Photomedicine at Massachusetts General Hospital (43–57) using photothermal [PT]-based (e.g., thermal lens and photoacoustic [PA]) and fluorescent methods, respectively. The advances in *in vivo* FCM were also made by other researchers and research groups (52, 58–79), including Irene Georgakoudi (Tufts University), Theodore Norris (University of Michigan), Xunbin Wei (Fudan University), Eric Tkaczyk (University of Tartu), and Anja Hauser (Deutsches Rheumaforschungszentrum). These advances were made mostly in the fluorescent-based *in vivo* FCM (58, 61–64,80), confocal fluorescence (59,60,62) backscattering (65,66) microscopy, multi-photon laser scanning microscopy (63,67–78), and coherent anti-Stokes Raman scattering (CARS) (79) techniques. Gold nanostructures were proposed to be used as scattering contrast agents *in vivo* FCM (80–81).

The adaptation of FCM principles from *in vitro* technology with cells flowing in well-controlled single file, to an *in vivo* approach using the blood vessels as natural tubes with native cell flows faces many challenges to *in vivo*. Cell velocity in blood, and especially in lymph flow, is much slower (from 0.5–5 mm/s in blood microvessels to 0.1–0.5 m/s in large blood vessels of animals and humans) than the maximum speed of analysis achieved in FCM *in vitro* (see above), which from this perspective makes FCM's technical platform easy to adapt to an *in vivo* setting. However, *in vivo* studies may be limited by 1) poor optical conditions (compared to an almost ideal situation *in vitro*), such as absorption,

scattering and autofluorescent background from the vessel wall, surrounding tissues (e.g., skin layers, connective tissue, muscles, or fat), or from blood cells; 2) multiple-file cell flow in vessel cross-sections; 3) difficulties of noninvasively accessing deep vessels; 4) problems with the use of a transillumination (forward) or sideways optical schemes, which is more convenient for achieving high spatial resolution; 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) compared to the well-controlled flow parameters of *in vitro* FCM. These limitations required some precautions in the choices of a detection system and proper animal models as a first step toward transitioning this technique to human applications. This can be done taking into account circulation properties and features. Significant contribution in developing optical methods for *in vivo* imaging of blood and lymph flow, measuring flow velocity, and study of circulation at different conditions has been made by Valery Tuchin's group in Saratov State University (SSU) starting since 1994 (82–99). It included the speckle techniques for cell velocity measurements in mostly used rat mesentery as animal model and vessels of eye conjunctiva in humans. In particular, complex dynamics of cell flows in lymph and blood vessels was studied, including the reactivity of lymphatic microvessels on topical application of a number of biologically important substances different in their nature, mechanisms, and action intensity, such as staphylococcal exotoxin (α-hemolysin), N-nitro-L-arginin (L-NNA), dimethyl sufoxide (DMSO), glucose, glycerol, etc. It was shown that nitric oxide (NO) plays an important role in the lymphatic microvessel regulation (94). One of the possible mechanisms of low-intensity laser therapy fundamentally based on NO signaling and regulating the drainage function of lymph microvessels was also proposed (96). The natural property of lymph vessel and valve function were studied in the detail (87, 94). The key principle investigator in this direction in Tuchin's group was Ekaterina Galanzha, who the further developed in Vladimir Zharov's group a video-imaging flow cytometry to make it faster and be applicable to blood flows, cell deformability quantification *in situ*, and cell-cell and cell-vessel wall interactions (100–112). A high-speed (up to 40,000 fps), high-resolution (up to 300 nm) continuous *in vivo* optical imaging technique (106) allows one to monitor and identify red blood cells (RBCs), white blood cells (WBCs), and platelets in the blood flow of rat mesenteric microvessels and other animal models without conventional labeling. It was demonstrated that the frame rate up to 10,000 fps at the high optical resolution could be sufficient for full estimation of individual cell behavior and cellular biomechanical properties *in vivo* in microvascular net. Cell focusing *in vivo* in native lymph flow was demonstrated using lymph valve as a natural nozzle (26).

A few other groups and researchers, including Stephen Morgan (University of Nottingham), Vyacheslav Kalchenko (Weizmann Institute of Science), and Gerard L. Coté (Texas A&M University) made a brilliant input in the problem of the label-free imaging of blood flow and measurement of cell velocity and oxygenation (16,17, 111–112, 113–119), which are partly summarized in (110) and presented in this issue (17).

Basic principles and detection methods

In FCM techniques individual cells of interest in blood or lymph flows are imaged with a sufficient rate and resolution by a CCD or CMOS camera using conventional microscopic lamp illumination and/or irradiated with one or several laser beams. Laser-associated physical effects in individual cells such as absorption, fluorescence, elastic and inelastic (Raman) scattering, PT and PA phenomena (Fig. 1) are detected with corresponding optical or non-optical detectors depending on the method used. Opto-physical time-dependent phenomena are generated in a cell via interaction with pulsed or intensity modulated optical radiation at different wavelengths (multicolor FCM).

Label-free video-imaging

The principle of this *in vivo* FCM method is illustrated in Fig. 1 and demonstrated in Fig. 2. A video-recording using a high-resolution and high-speed CCD or CMOS cameras in transmittance or reflectance mode allows one to get pretty good cell images in lympth or blood flows. As an example, high-speed transmittance digital microscopy (TDM) images of cells in the area close to valve of the lymph vessel of rat mesentery demonstrating the natural cell flow hydrodynamic focusing are shown in Fig. 2. A vein (or lymphatic) valve works as a nozzle, it is like a natural hydrodynamic cell used in conventional *in vitro* FCM allowing for cell flowing one by one.

Potential and already realized applications of a high-speed TDM include (19,35,100–112): fundamental study of cell–cell interactions in native flow; estimation of the proportion of fast-moving WBCs traveling with RBCs in axial flow to slow-moving (rolling) WBCs, which may have diagnostic value for the study of some pathologic conditions; identification of rare abnormal cells (e.g., cancerous or sickled cells) on the basis of their different deformability in flow; imaging of platelets during thrombus formation and interaction with metastatic cancer cells; study of the impact of drugsorradiation on individual cells; estimation of blood viscosity in a high-velocity flow; study of cell aggregation dynamics and adhesion to endothelial cells; estimation of velocity profiles in fast flow; and imaging and detection of individual cell dynamics in afferent lymph flow with cell velocity up to 7 mm/s.

As mentioned, an exposure of the vessel by a laser light induces a number of physical phenomena each of which give rise to a unique cytometry techniques such as light scattering, light transmittance, fluorescence, Raman, PT, or PA methods (Fig. 1), where TDM serves also as a navigator instrumentation for laser beams and finding the region of interest of the vessel net for cytometry *in situ* using relatively thin transparent tissue.

PT and PA methods

These methods are based on nonradiative relaxation of absorbed laser energy into heat and accompanying acoustic effects (Fig. 1,3). Among different PT-based methods (120, 121), time-resolved two beam (pump-probe) PT thermal lens detection and imaging methods with one (121) and multiplex (122) channel schematics was mostly used in *in vivo* FCM. In PA FCM, cells in blood or lymph flow are irradiated with a focused laser beam and laser-induced PA waves (referred to as PA signals) are detected with an ultrasound transducer attached to the skin (Fig. 3). These methods offer the highest absorption sensitivity at the single-cell level, 10^{-2}–10^{-4} cm^{-1}. This level of sensitivity enables imaging of cells noninvasively (i.e., with a short-term temperature elevation ≤0.5°C), with molecular specificity based on label-free intrinsic absorption spectroscopic contrast (e.g., from hemoglobin, melanin, or cytochromes) or on low-toxicity, functionalized strongly absorbing nanoparticles (NPs) (Fig.3) PA technique demonstrates an advantage in assessing of deep vessels *in vivo* (up to 1–3 cm) (123) while PT methods have higher sensitivity in the study of relatively transparent thin structures like animal ear or mesentery. Recent technical advances include use a high pulse repetition rate nanosecond lasers (up to 0.5 MHz) such as diode laser at 905 nm (29) and fiber-based Yab laser at 1064 nm (34,40), two-color PA FCM (15, 26,29, 41), integration of *in vivo* magnetic enrichment and multiplex PA detection (15), minimally invasive fiber delivery of laser radiation to vessels (29,30), and potential to use contrast agent with tunable ultrasharp PT and PA spectral resonances and holes (up to a few nm width) for multicolor detection (124).

Fluorescent methods

Fluorescence detection schematics were used in *in vivo* FCM in different modifications (e.g., confocal or two-photon) employing standard fluorescent labels as in conventional

FCM *in vitro* (1). In the confocal scheme, fluorescent signals from the cell populations of interest were recorded as the cells passed through a slit of laser (e.g., He-Ne) light focused across 20–50-μm mouse ear blood vessels (43,44). Emitted fluorescence was collected by microscope objectives, and directed through a dichroic splitter and mirrors to photomultiplier tubes. Compared to single-photon fluorescence microscopy, multiphoton fluorescent technique (68,76) can increase the depth of light penetration in microvessels located deeper in tissue (few hundred μm) and reduce out-of-focus photodamage. However, this technique is typically using focused circle laser beams (compared to classic linear beam shape) that may lead to missing cells flowing in relatively large vessels outside irradiated zone. Recent advances include use of two color schematics (49,71) and fiber-based deliverylaser light to deep vessels (75).

Raman spectroscopy

Zharov's team introduced *in vivo* Raman flow cytometry in which scattering signals from Raman active vibrational states were detected using a high sensitive optical technique with fast spectral acquisition down to few milliseconds (28). This technique can use either intrinsic contrast agents such as lipids with CH_2 state or exogenous labels such as carbon nanotubes and as proposed in (6,7 33) surface-enhanced Raman scattering (SERS) agents including gold nanoparticle coated with Raman active dyes.

An integrated Raman-based cytometry was developed with PT and PA two-beam detection of Raman-induced thermal and acoustic signals in biological samples with Raman-active vibrational modes (33) that can be applied also for *in vivo* FCM. Coherent anti-Stokes Raman scattering (CARS) demonstrated promises for detection of tumor cells with high lipid content (79).

Detection of the scattered light

In conventional *in vitro* FCM, detection of forwardly and orthogonally scattered light allows for discrimination between lymphocytes, monocytes, and granulocytes (1). Light scattering spectroscopy has identified differences in the wavelength dependence of backscattered light between normal and cancer cells due to cellular transformations in early stages of disease development. Recently the backscattering confocal systems allows for characterizing differentially leukemic and normal WBCs and RBCs were developed (65,66). This approach has good perspectives for *in vivo* light scattering FCM.

To reduce the interference from light scattering background from surrounding tissue and normal cells, gold NPs with strong light scattering due to plasmonic resonance was proposed as contrast agents for *in vivo* FCM (80,81). Further development of *in vivo* scattering FCM may include detection of enhanced scattering effects from laser-induced nano- and microbubbles around overheated absorbing contrast agents as demonstrated *in vivo* for non-moving cells (125).

Cell flows

Most studies were performed on superficial ~50 μm blood microvessels in mouse ear. PA FCM with ability to assess deep vessels demonstrated noninvasive detection of CTCs in ~300 μm skin vessels and 0.9 mm aorta at depth around 2–3 mm using focused cylindrical ultrasound transducer (29). Zharov's team extended application of *in vivo* FCM on lymph flow using natural cell focusing after lymphatic valves (19, 22, 26) and recently on bones (36) and plants (37, 126).

Labeling in vivo

The great advantage of *in vivo* FCM is the possibility for label-free detection using intrinsic contrast agents, such as hemoglobin, melanin, or cytochromes, in video-imaging and PT/PA methods (19,20, 23) or lipids in Raman-based techniques (79), and intrinsic scattering (1, 65,66) or autofluorescence properties of cells. Nevertheless, cells can be labeled directly in the bloodstream by intravenous injection of florescent dyes (43, 46, 57) of functionalized NPs (Fig.3) (15). Depending on cell and contrast agent types labeling procedure on mice models takes from 10–20 min (15) to approximately one hour (57).

Animal models and potential for humans

To fully exploit the capabilities of FCM, it is extremely important to choose the proper animal model. The acute preparation is widely used and usually requires a surgical procedure on the anesthetized animal while placing it on a specially heated platform (22, 96, 109). Such preparations are usually required for studying the microcirculation of internal organs, tumor development, angiogenesis, inflammation, thrombosis, and the progression of infection. FCM utilizes acute preparations in rodent models to expose the vessels of mesentery, skin flap, bone, brain, liver, kidney, lungs, and cremaster muscle (109,118). Less invasive are models of eye conjunctiva (99), iris (119) and retina (59,60) which are directly applicable in humans. A widely spread and intensively developing human noninvasive model is the nailfold model, which is on use in *in vivo* capillaroscopy for many years (16, 17).

Many studies *in vivo* FCM were performed in thin (250–300 μm), relatively transparent (i.e., also suitable for PT FCM (in transillumination mode) mice ear with well-distinguished blood microvessels. The ear blood vessels under examination are located 30–100 μm deep and have diameters in the range of 30–50 μm and blood velocities of 1–5 mm/sec (23,43). Also many experiments were performed with the rat mesentery which has an almost ideal vascular and surrounding tissue structure to proof concept of *in vivo* FCM because it consists of very thin (7–15 μm) transparent connective tissue with a single layer of blood and lymph microvessels (109).

These animal models were successfully used in various promising applications (see below) at fluorescent *in vivo* FCM. However, translation of this technology to *in vivo* situation in human faces many challenges such as toxicity of fluorescent probes, broad emission spectra in near-infrared (NIR) window of tissue transparency (i.e., multicolor problem), strong scattering and autofluorescence background, high intensity of CW laser used (10–100 W/cm^2) compared to laser safety standard (0.2 – 0.5 W/cm^2) (127), and assessment only superficial (≤ 0.3 mm) microvessels with a slow flow rate. For example, in 50-μm blood vessel with flow velocity of 5 mm/s, approximately two days are required to continuously assess total ~2-ml blood mouse volume.

PT and, especially PA, methods are almost free of these limitations. For example, optical-resolution photoacoustic microscopy (OR-PAM) working in reflection-mode is described (128). It allows for *in vivo*, noninvasive, label-free, three-dimensional (3D) microvascular imaging down to single capillaries and quantification of total hemoglobin concentration and hemoglobin oxygenation saturation. Several successful pilot trials on humans demonstrated a clinical significance and safety of PA technique including: 1) continuous monitoring of blood oxygenation in the internal jugular vein (10–20 mm in diameter, 5–10 mm deep) in the presence of strong light scattering and attenuation in the 15–20 mm layer of overlying tissue (129); 2) detection and imaging of breast tumor at a depth of 3 cm (130); and 3) monitoring of blood hemoglobin in the wrist area overlying the radial artery at a depth of 3 mm (131). PA technology is safe for human subjects as it requires a laser energy fluence of

only 5–20 mJ/cm^2 which is well within the laser safety standard (35–100 mJ/cm^2) in NIR range (e.g., 700–1100 nm) (127). The high sensitivity of nonlinear PT/PA technique based on synergistic amplification of PT/PA signals associated with high localized (within single cells) laser-induced nanobubbles around overheated multilayer (e.g., with ethanol) plasmonic NPs, and especially their clusters allows further reduction the threshold of laser pulse fluence to 1–5 mJ/cm^2 (32,36, 132–136). First clinical prototype of fiber-based PA FCM is shown in Fig. 4 (29,41).

Applications

The status and potential applications of different modification of in vivo FCM are discussed in previous (5,22,25–27) and presented in this issue's papers. Most studies were performed on 50–300 μm blood microvessels with cell flow rate in the range of 10^4 – 10^6 cells/s (i.e., comparable or higher than in conventional in vitro FCM with typical cell rate of 10^4 –10^5 cell/s) with focus on detection of relatively rare cells with linear laser beam shape overlapping vessel diameter. Briefly, the capacity of clinically relevant in vivo PA FCM technologies was demonstrated by the real-time detection in blood and lymph flows of circulating individual normal cells (e.g., erythrocytes and leukocytes) in different functional states (e.g., normal, apoptotic, or necrotic), tumor cells (melanoma, breast, squamous), bacteria (e.g., *E. coli* and *S. aureus*), NPs (e.g., gold nanorods, carbon nanotubes, magnetic and golden carbon nanotubes, and dyes (e.g., Lymphazurin, Evans Blue, and Indocyanine Green) (15, 19–42). Compared to other promising approaches PT FCM and PA FCM offer the following advantages: 1) use of low-toxicity NPs approved for a pilot study in humans; 2) high-resolution (300 nm) PT and optical imaging (*in vivo* image FCM) of flowing single cells of interest at velocities up to 2 m/s (23,34,40); 3) PT and PA measurements of the velocity of individual flowing cells (19, 29,42) and of cell flows using optical speckle measurements (96, 97, 99); 4) theranostic of circulating abnormal cells (e.g., CTCs) as integration of PA diagnostics, real-time highly localized (i.e., not harmful for surrounding normal blood cells) PT killing using the same laser, and control efficiency of therapy through decrease PA signal amplitudes and CTC counts (29, 31); 5) PA detection of disseminated tumor cells in lymphatics as the earliest prognostic marker of metastasis, compared to sentinel lymph node and blood assessment (30); 6) use of magnetic NPs as multifunctional PA-PT-magnetic resonance imaging contrast agents for *in vivo* magnet-induced NP clustering, nanobubble-related signal amplification, imaging, and cell enrichment/sorting/separation (15, 31); and 7) the use of PA FCM both with noninvasive transcutaneous laser irradiation and minimally-invasive approach using tiny needle or catheter for delivery of laser radiation theoretically in any vessels inside the body (25, 29). Recent applications include real-time monitoring of blood rheology parameters and its change during pathological processes (review in this issue [38])], label-free detection of circuiting clots using negative PT and PA contrasts for early cardiovascular disease diagnosis and potentially prevention stroke (this issue [39]), *in vivo* real-time monitoring of dye-cell interaction, circulating dead cells, and potential blood volume (this issue [41]), (and cell identification based on their different velocity in circulation (42). One of the most important clinical applications in future should be high sensitive detection of CTCs (melanoma and breast) with unprecedented threshold sensitivity as 1 CTC/mL on animal model with potential to improve this threshold by two orders of magnitude on humans (15, 39), detection of the extremely rare circulating cancer stem cells (31), and dangerous clots (39).

In the initial study of Lin's group fluorescent detection mode was used (43–48). RBCs were isolated from the animal, fluorescently labeled *ex vivo*, and then reinjected into the animal. In contrast, WBCs were labeled *in vivo* by injecting a WBC surface antigen-specific antibody. It was observed that the number of circulating RBCs remained constant for at least

3 days, while the number of WBCs decreased significantly in a few hours compared to the much longer life of non-labeled cells. This finding resulted possible from the influence of the tags on genuine cell interactions in flow. These experiments also revealed the dependence of the kinetics of circulating cancer cells, in particular, the dependence of the rate of cell elimination from blood flow, on the type of cancer cells and the host environment (44). Fluorescent techniques in animal models have shown promises for detecting labeled hematopoietic stem cells and green fluorescent protein–expressing cells (49, 52, 55). The methods used to label circulating cells for fluorescence detection by *in vivo* FCM which are useful for cell tracking in small animals, and are particularly useful for the detection of circulating cancer cells and quantification of circulating immune cells are overviewed in this issue (57).

No doubts that *in vivo* FCM is currently a fast growing area of research with large horizon of new opportunities in biological sciences and medical fields including combination of different detection methods (21,22).

This special issue is mostly focused on the state-of-the-art in the field of *in vivo* FCM and its applications with particular emphasising the biophotonic methods, disease diagnosis, and monitoring of disease treatment at single cell level in flow conditions. The use of photonic technologies in medicine is a rapidly emerging and potentially powerful approach for disease detection and treatment. Therefore, the collection of papers from the leading groups in the field seeks to advance scholarly research that spans from fundamental interactions between light, cells, vascular tissue, and labeling particles, to strategies and opportunities for preclinical and clinical research. The general topics are fast video-imaging microscopic, polarization sensitive, laser-scanning, light scattering, confocal microscopic, single and multiphoton fluorescence, PT and PA FCM methods for cellular diagnostics and monitoring of disease treatment in living organisms and plants.

It is opened by a few comprehensive reviews by Galanzha and Zharov on the analysis of the capability of PT and PT FCMs for monitoring multiple blood rheology parameters including identification of sickle cells in a mouse model of human sickle cell disease (38), by Pitsillides et al. on the analysis of cell labeling approaches for fluorescence-based *in vivo* FCM (57), by Morgan on optical techniques for *in vivo* imaging of the microcirculation and FCM with focus of its capability to assess sickle cells (17), by Tkaczyk ER and Tkaczyk AH on strategies and applications of multiphoton FCM (76), by Niesner and Hauser on recent advances of dynamic intravital multi-photon microscopy (78), and Wlodkowic et al. on innovative technologies for *in situ* analysis of small multicellular organisms (137). Most reviews contain carefully selected and deeply analyzed data which are typically summarized in the convenient way for readers mostly in well-designed tables. The table with listed methods of cell labeling for *in vivo* FCM as well as the cell types studied, can be found in paper (57). The table summarizing optical techniques for *in vivo* imaging of the microcirculation, such as multiphoton microscopy (MPM), second harmonic generation (SHG), fluorescence lifetime imaging microscopy (FLIM), CARS, Doppler optical coherence tomography (DOCT), optical microangiography (OMAG), PA microscopy (PAM), PT microscopy, laser speckle contrast analysis (LASCA), and hyperspectral imaging (HIS) is presented in paper (17). Paper (76) contains a table with overview of cytometry variations incorporating multiphoton excitation, including techniques that have been used *in vivo*. Imaging techniques, their lateral and axial resolution, imaging depth, intravitality/*in vivo* (cell culture/organ models) investigative capabilities are summarized in the table of paper (77). The following techniques are analyzed: MRI, PET/CT, PA microscopy, optical coherence tomography (OCT), wide-field microscopy, confocal microscopy, multi-photon microscopy, confocal or multi-photon endoscopic microscopy, 4Pi-microscopy, stimulated emission depletion microscopy (STED-microscopy), stochastic

optical reconstruction microscopy (STORM), photo-activated localization microscopy (PALM) and some others.

The remaining papers are original ones: they demonstrate the cutting edge of research and developments in *in vivo* FCM (37, 39–41, 62) and present techniques prospective for *in vivo* and microfluidic-based FCM (65,66). Four original papers are from Zharov's group. The paper by Nedosekin et al. (40) demonstrates the ultra-fast *in vivo* PA FCM of circulating human melanoma cells either with label-free detection or with molecular targeting and magnetic enrichment using conjugated magnetic NPs; paper by Proskurnin et al. (41) shows potentialities of PT and PA FCM with multicolor dyes for *in vivo* real-time assessment of circulation, dye-cell interaction, and blood volume; the paper by Galanzha et al. (39) presents successful application of PT and PA negative contrasting for cytometry of *in vivo* circulating clots; and paper by Nedosekin et al (37) proposes the real-time noninvasive FCM for study of transported nanomaterials in xylem and phloem plant vascular systems.

One paper by Li et al. (62) is from Wei's group, where authors using fluorescence *in vivo* FCM quantified the number and flow characteristics of labeled cells and on this basis investigated the relationship between metastatic potential and depletion kinetics of circulating tumor cells. The two last papers from Georgakoudi's group by Greiner et al. (65,66) describe in detail fundamentals, instrumentation and applications of confocal backscattering spectroscopy for leukemic and normal blood cell discrimination in flowing blood samples.

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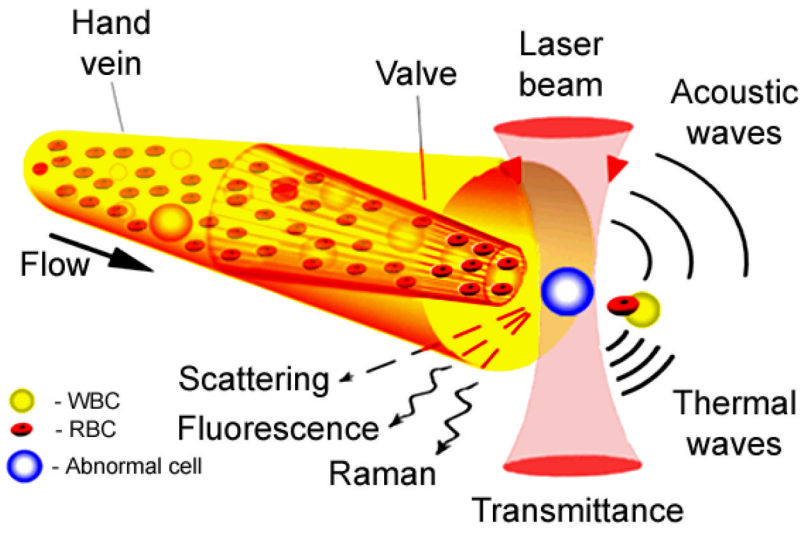

Fig. 1.
Principle of in vivo flow cytometry integrating multimodal detection of individual circulating cells directly in the bloodstream or lymph flow.

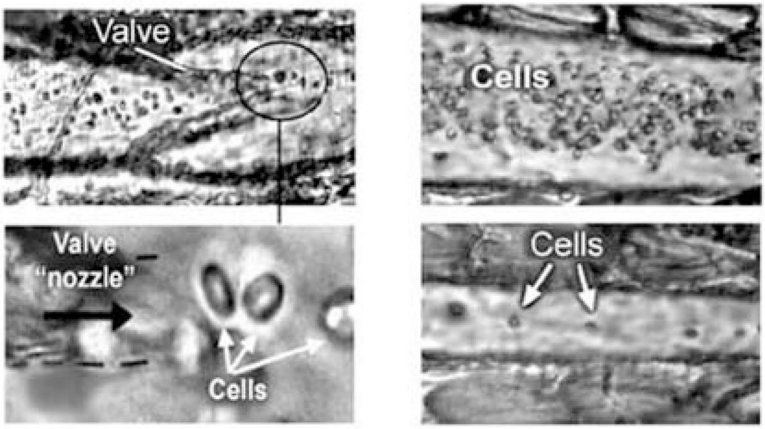

Fig. 2.

High-speed transmittance digital microscopy (TDM) images of cells in the space within the valve area (left) and immediate after a valve (right) of the lymph vessel of rat mesentery at ×10 (top) and ×100 (bottom) magnifications demonstrating the principle of cell flow hydrodynamic focusing. TDM images of cells in the central part of a lymphangion in diastole (top, right) and systole (bottom, right) phases are also shown [from (26)].

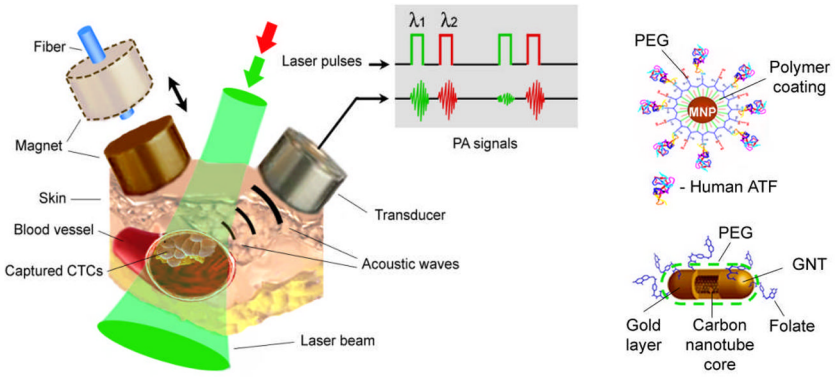

Fig.3.

In vivo magnetic enrichment and multiplex two – color photoacoustic (PA) detection of circulating tumor cells (CTCs) molecularly targeted by cocktail of golden carbon nanotubes (GNTs) and magnetic nanoparticles (MNP) [from (15)].

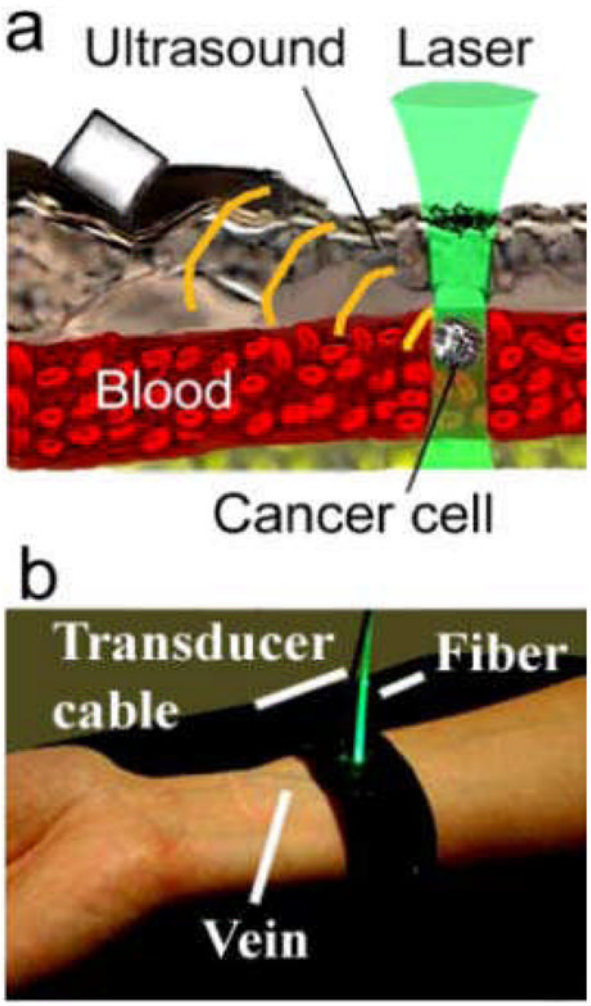

Fig.4.
(a) Schematic of in vivo PA flow cytometry (FCM). (b) Clinical prototype of personal *in vivo* PA FCM (from [29,41]).