OPEN ACCESS
Journal of Innovative Optical Health Sciences
Vol. 16, No. 3 (2023) 2330005 (11 pages)

DOI: 10.1142/S1793545823300057

World Scientific
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Detection of cells by flow cytometry: Counting, imaging, and cell classification

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Received 31 July 2022
Accepted 15 February 2023
Published 31 March 2023

The study of circulating cells in the blood stream is critical, as it covers many fields of biomedicine, including immunology, cell biology, oncology, and reproductive medicine. *In-vivo* flow cytometry (IVFC) is a new tool to monitor and count cells in real time for long durations in their native biological environment. This review describes two main categories of IVFC, i.e., labeled and label-free IVFC. It focuses on label-free IVFC and introduces its technological development and related biological applications. Because cell recognition is the basis of flow cytometry counting, this review also describes various methods for the classification of unlabeled cells, including the latest machine learning-based technologies.

Keywords: *In-vivo* flow cytometry; label-free; cell classification.

1. Introduction

Cells circulating in the blood stream can hold signatures of anomalies or dysfunctionalities of several body organs, which could indicate various infections or diseases. Therefore, the capability to detect and quantitatively analyze circulating cells can be a valuable asset in furthering the research on diseases and early diagnosis of cancer. Conventional flow cytometry (FC) has been widely used in biomedical and clinical applications as a general tool for *in-vitro* detection of peripheral blood cells.[1–3] In FC, blood is drawn from a host and the cells of interest

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are labeled. When cells pass individually through a sheath flow, they are irradiated by a laser. Then, the emitted florescence and the forward and side scatter from the cells are collected by multiple photomultiplier tubes (PMTs), which can provide information on various parameters related to these cells.

However, in the conventional FC, cells are detected *in vitro*, which presents several limitations as follows[4,5]:

(1) The process of drawing blood from the host organism has been shown to trigger a stress response.[6] It may further induce changes to the cell properties or cause cell death. Moreover, this *in-vitro* detection is not conducive to the evaluation of cell metabolic activity in real and complex physiological environments.
(2) The small volume of the drawn blood may limit the sensitivity of cell detection. The blood volume is often insufficient to count rare circulating cells in the blood, especially circulating cancer cells in blood levels of 10–100 ml.
(3) FC cannot provide real-time detection of cells because the samples need to be processed, which is time consuming. Moreover, the discontinuity of sampling is not conducive to long-term monitoring of the cell changes.

To overcome these limitations, *in-vivo* flow cytometry (IVFC) was suggested to provide an intravital, noninvasive, and real-time cell detection method. In the IVFC method, the flow of the blood stream acts as the sheath flow. When labeled cells pass through the laser slit and get excited, a fluorescence signal is generated, which is then collected by the PMT.[7] Compared with the conventional FC, the new IVFC tool exhibits unique advantages. This study introduces a brief history of IVFC and describes its features using confocal and multiphoton microscopies as well as computer imaging, photoacoustic (PA), and other methods. This ensemble of approaches is classified into two major categories: Labeled IVFC and label-free IVFC. The methods in the first category (i.e., labeled IVFC), characterized by high contrast and high sensitivity, have been extensively studied and reviewed in the literature.[7–9] The methods in the second category (i.e., label-free IVFC), featuring noninvasive and nontoxic methods, are the focus of our review. The application of these methods to the classification of unlabeled cells, including that using recent deep learning-based technologies, is discussed.

2. Labeled IVFC

The scattering, absorption, and refraction of the laser beam by the vessel walls and surrounding tissues constitute a challenge in IVFC. These phenomena lead to light attenuation and limit the depth of cell detection. To overcome these limitations, labeled IVFC can improve the contrast between the surrounding tissues and cells with fluorescent labeling, resulting in improved sensitivity of detection. In this section, we introduce the labeled IVFC based on fluorescence, and do not discuss the IVFC labeled with nanoparticles. Here is a summary of the methods of fluorescence labeling.[6,10,11]

The first method involves injecting a fluorescent probe into a recipient. The antibodies or antibody fragments bound to the fluorophore are injected into the circulating cells as targets of specific cell surface receptors. This method can easily detect cells in the cycle, but it suffers from several challenges. On the one hand, the antibody–antigen binding can easily deplete the cells during the cycle. On the other hand, cell surfaces can easily loose the fluorescence labeling, which diminishes the possibilities of performing long-term *in-vivo* monitoring.

The second method is based on the labeling of cells with fluorescent tracers *in vitro*. When the labeled cells are further injected intravenously back into the body (e.g., an animal body), the fluorescence brightness of the labeled cells will gradually decrease after many divisions. However, the fluorescent labeling in this method can remain in the cycle for a longer period, which enables the monitoring of cells for several weeks.

The third method involves encoding the fluorescent protein (FP) genes in the cells. As long as the FP gene is stably expressed in the cell, the fluorescence brightness will not decrease after cell division and can be monitored continuously *in vivo*. The expression of this fluorescent label is longer than that in the previous two methods and suitable for long-term monitoring of tumor growth.

2.1. *Microscope-based fluorescence IVFC*

2.1.1. *Confocal microscope IVFC*

Confocal microscopes focus light on a point or line and can improve the imaging quality by detecting pinholes, which filter out the imaging information beyond the focusing plane. Novak *et al.* developed

the first IVFC based on fluorescence confocal excitation in 2004.[12] In their setup diagram, the microscope objective lens focused a laser beam onto a selected artery of the mouse ear. When the labeled cells in the circulation flowed through the slit, the labels were excited by a He–Ne laser. After the fluorescence signal was collected by the PMT, the pulse signal was analyzed and evaluated offline using MATLAB (Fig. 1). In their study, the cells were labeled with red fluorescence at visible wavelengths by using DID and Cy-Chrome-CD45. Then, they verified the circulating kinetics of the cells, which proved the ability of IVFC in detecting living cells.

The initially developed IVFC had one channel and could detect fluorescence of only one color. To overcome this limitation, Novak *et al.* proposed a two-color, double-slit IVFC device, which could detect fluorescence of two different colors simultaneously.[13] The device included a 473 nm semiconductor laser and a 633 nm He–Ne laser to excite two different cell populations, each labeled with a different fluorophore. The two channels were also used to study one cell population with multiple molecular targets.[14,15]

In comparison with visible light dyes, near-infrared (NIR) dyes exhibit lower scattering and high penetration. In 2015, Suo *et al.* designed an NIR detection channel with a 785 nm laser to excite the circulating cells labeled with IR-780 dye.[16] The team quantified the representative peaks of NIR fluorescence, which means that the detection band of IVFC extended from the visible to the NIR range. Ding *et al.* proposed another method to improve the light penetration by using an optical clearing agent (OCA) instead of glycerol to make the rat ear skin more transparent, which improved the signal-to-noise ratio of the IVFC.[17]

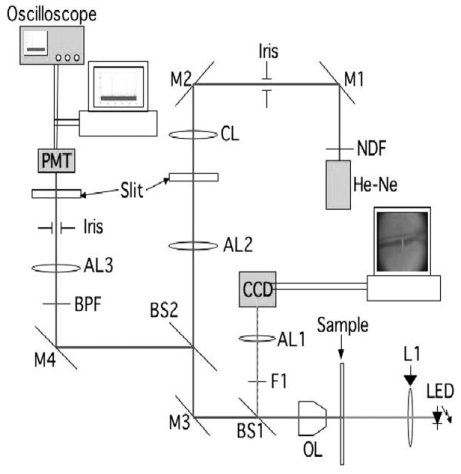

Fig. 1. Schematic of experimental setup of *in-vivo* flow cytometer: L1, condenser lens; OL, microscope objective lens (40×, 0.6 numerical aperture, infinity corrected); BS1, BS2, dichroic beam splitters; AL1–AL3, achromats; CL, cylindrical lens; M1–M4, mirrors; NDF, neutral-density filter; BPF, bandpass filter; PMT, photomultiplier tube. (Reproduced from Ref. 12.)

Although the sensitivity of IVFC for rare circulating cells can be improved through long-term monitoring when compared with FC, strong focusing provides a very small detection area, and the blood flow detected remains relatively small. In 2007, Lin *et al.* designed retinal flow cytometry (RFC), which is essentially a confocal scanning microscope.[18] By using two resonant scanners to quickly obtain a circular scan of the optic nerve head, RFC can monitor multiple pairs of blood vessels simultaneously. This greatly increases the number of counted cells, thus improving the detection sensitivity.

2.1.2. *Multiphoton IVFC*

Another instrument commonly used in IVFC is the multiphoton microscope, which uses an infrared light source of longer wavelength. Therefore, it is less affected by light scattering and can easily penetrate the sample tissue. In 2007, He *et al.* proposed an improved multiphoton microscope with one-dimensional line scanning for sensitive counting of rare circulating tumor cells (CTCs).[19] In 2008, Tkaczyk *et al.* reported dual-channel two-photon FC to monitor multiple tumor cell populations simultaneously in the cycle.[20] In 2010, Chang *et al.* improved the device by using a double-clad optical fiber as a probe, which avoided the scattering and absorption by the blood.[21] Combined with fluorescence labeling, this method achieved high efficiency and high sensitivity for the detection of circulating cells. In 2016, Kong *et al.* introduced multi-color, multiphoton *in-vivo* imaging FC, which integrated an optical phase-locked ultrasound lens with a standard TPM.[22] The proposed system was used for fast volumetric imaging of the cellular dynamics in the bone marrow without thinning the skull.

2.2. *Other fluorescent IVFC methods*

In addition to microscope-based fluorescent IVFC, several typical fluorescent IVFC methods are

introduced here, including diffuse fluorescence IVFC, computer vision IVFC, and PA and fluorescent IVFC.

In 2012, Zettergren *et al.* developed a new instrument, called diffuse fluorescence flow cytometer (DFFC),[23] which illuminates the relatively narrow (2–3 mm) and small limbs of mice with a 642 nm continuous-wave laser. The advantage of DFFC is that the detection site involves many pairs of high-speed blood vessels, which greatly shortens the time of detection and improves the sensitivity. The team also developed a new fiber-based optical probe to eliminate artifacts caused by breathing, which improved the signal-to-noise ratio.[24] On the basis of previous studies, the team adopted a dual-fiber optic probe and a new pairwise coincidence detection algorithm, which almost eliminated the false positive signals caused by electronic noise and motion.[25] The new setup was able to measure the velocity of the cells labeled with a NIR dye. This method allowed the entire blood of mice with 2 mL circulation volume to be detected within 10 min.

In 2013, Markovic *et al.* designed a computer vision IVFC method,[26] which could image three to four pairs of large blood vessels in a rat ear with a relatively large imaging area ($\sim 5 \times 5\,\mathrm{mm}^2$), thus greatly improving the sensitivity of detecting the circulating cells. Their paper described the combination of a macroscopic fluorescence imaging system (collecting fluorescence image sequences with a high-sensitivity electron-multiplier charge coupled device camera) and an automated computer vision algorithm. The algorithm first identifies the candidate cells in a single frame, and then merges them into tracks based on dynamic analysis of the image sequence. On the basis of a previous study, the team used weak fluorescence markers to verify that the instrument maintained a sensitivity of at least 50% even when the contrast was reduced by two orders of magnitude.[27]

In 2014, Nedosekin *et al.* used *in-vivo* PA and fluorescent flow cytometry[28] to monitor the changes in the CTC levels of mice during a tumor operation.[29] In the PA channel, CTCs were detected among red blood cells (RBCs) owing to the higher light absorbance. In the fluorescence channel, they were detected among RBCs owing to the brighter labeled fluorescence when compared with autofluorescence.

3. Label-free IVFC

The purpose of label-free IVFC is to provide long-term noninvasive, nontoxic, and real-time monitoring of a receptor, which reduces the effect of fluorescent markers on living organisms and the experimental errors caused by individual differences; thus, it is more suitable for clinical research *in vivo*. Label-free IVFC does not rely on exogenous markers to provide contrast. Instead, it realizes cell imaging and detection based on certain characteristics of the substances in the tissue (endogenous contrast, spectral characteristics, etc.), which can provide the morphological and biochemical information of the cell.

3.1. *Photoacoustic IVFC*

The principle of PA technology is based on the thermal expansion of tissues or cell structures after absorbing laser energy. As shown in Fig. 2, laser-induced PA waves, referred to as PA signals from the cells in the blood (or lymph) flow, are detected using an ultrasound transducer attached to the skin.[30] The method with nonfluorescent labeling can maintain good cellular characteristics and cellular physiological function without photo bleaching, light flickering, and cytotoxicity. Zharov *et al.* were the first to develop NIR photoacoustic flow cytometry (PAFC).[30–32] The He–Ne and Nd:YAG lasers focused on single cells in the blood or lymphatic flow in melanoma-bearing mouse, and ultrasonic transducers were used to detect the PA signals. The repetition rate of the laser pulse in the first PAFC was only 10 Hz, which had limited capability of detecting cells in fast-flowing blood vessels. Therefore, the authors proposed an improved PAFC with

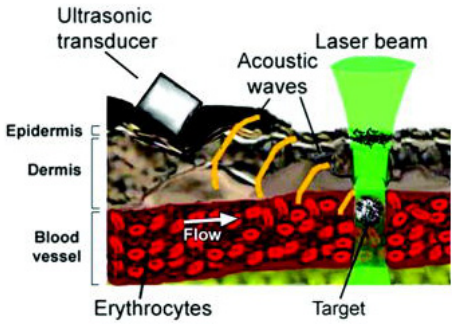

Fig. 2. Principle of PA detection of single absorbing targets in blood flow *in vivo*. (Reproduced from Ref. 30.)

a high pulse-repetition-rate diode laser (905 nm, 0.01–0.7 J/cm^2), which could achieve a maximum pulse repetition rate of 50 Hz, thus yielding ultrasensitive detection.[33] The team also developed a PAFC method based on Yb-doped fiber laser with high pulse energy up to 50 μJ at 1064 nm, which could detect PA signals from rare CTCs in large blood vessels.[34]

3.2. *Spectrally encoded flow cytometry (SEFC)*

Spectrally coded imaging is a label-free, high-speed imaging technique that can visualize fast-flowing blood cells in the body. In 2012, Golan *et al.* proposed SEFC, in which a broadband light with central wavelength of 840 nm and spectral bandwidth of 50 nm was focused onto a transverse spectrally encoded line using transmission diffraction grating and a water immersion objective lens. When cells flow unidirectionally in a blood vessel, the reflected light can be coupled with an optical fiber with confocal pinholes and collected by a spectrometer equipped with a high-sensitivity CCD.[35] Because the SEFC does not need a mechanical scanning probe, the imaging efficiency is increased; thus, this method can be used to image cells with a high acquisition rate and high resolution. In recent years, SEFC has achieved high-speed, high-throughput blood vessel imaging,[36] hematocrit measurement, and classification of leukocyte subtypes.[37] In 2022, an analytical model was established to describe the morphology of RBCs lacking light-scattering organelles, which is helpful in extracting important clinical parameters from the raw images of SEFC.[38]

3.3. *IVFC using two-photon excited fluorescence imaging*

Two-Photon excited fluorescence (TPEF) is a representative method for *in-vivo* label-free cell imaging and counting that relies on the excitation of autofluorescence. Two-photon excited autofluorescence is a noninvasive, high-resolution, high-penetration optical microscope with unique advantages in label-free detection of biomolecules and cells. By utilizing the low average energy, a high-pulse-energy picosecond or femtosecond NIR pulsed laser can provide high detection sensitivity as well as reduce the phototoxicity of the sample. In 2012, Zeng *et al.* used TPEF to excite strong autofluorescence of plasma and nicotinamide adenine dinucleotide (NADH) signals of white blood cells (WBCs) in zebrafish to improve the endogenous contrast, which enabled the visualization of the blood flow and counting of individual RBCs and WBCs.[39] In 2017, Bower *et al.* used two-photon excited fluorescence lifetime imaging microscopy (TEP-FLIM) to observe the binding dynamics of endogenous fluorophore NADH in hairless mouse epidermal keratinocytes for tracking the metabolic process of apoptosis.[40,41] TPEF can overcome the difficulties in realizing imaging or counting of individual cells in the blood flow and provides good contrast for potential imaging of various blood cells noninvasively. However, the limited number of discovered endogenous fluorophores limits the cell types to which the method can be applied. Another limitation of this method is that it is almost impossible to detect the same cell again in another measurement.[42]

3.4. *Other label-free IVFC methods*

In addition to PAFC, some label-free IVFCs can provide morphological and biochemical information of samples.

In 2017, Zhang *et al.* demonstrated an absorption intensity fluctuation modulation (AIFM) effect based on the absorption difference between the RBC and background tissue. Full-field functional optical angiography and blood cell counting could be realized by separating the dynamic and static signals in the frequency domain and calculating the real-time modulation depth. In addition, the physiological mechanisms of the blood circulation systems were monitored and diagnosed.[43]

In 2017, Chen and Huang developed a transient absorption microscope to measure the flow velocity of unlabeled RBCs *in vivo*.[44] Based on the property that RBCs can exhibit intrinsic transient absorption in the NIR region with little interference from the external environment, the team used a pump beam of 1064 nm and a probe beam of 810 nm to perform U-shaped repetitive scanning of zebrafish capillaries, and obtained high-resolution images for three-dimensional reconstruction of the zebrafish vessel network.

3.5. *Living cell classification via label-free IVFC*

Blood cell detection and classification results have become an important reference for modern medical diagnosis of the health status of patients. The results of blood cell classification can provide the basis for cell counting in FC. Unlike fluorescent IVFC, which realizes cell classification by labeling, several unlabeled cell classification methods are available, as introduced here (Table 1).

Based on the characteristics of endogenous absorption of cells, He *et al.* proposed a PAFC with fast B-scan imaging of the cells.[45] Through a comprehensive analysis of the size and shape of the cells in the image and the contrast of 532 nm laser absorption, they could identify melanocytes and RBCs in the mouse model.

Autofluorescence can be utilized for *in-vivo* imaging of blood vessels or cells, as demonstrated by several research groups, and cells can be identified according to their morphology and expression characteristics. For example, based on the color coding of the different spectral characteristics and fluorescence lifetimes of hemoglobin and tryptophan in blood, Li *et al.* distinguished between the red and WBCs in the microvessels in the hamster pouch.[46] In 2012, Zeng *et al.* used the different endogenous fluorescence contrasts of zebra fish vascular substances to distinguish between red and WBCs.[39]

Based on the differences in the spectral characteristics of different blood cells, several research groups have carried out experimental studies on *in-vivo* imaging with different spectra and classified the blood cells. In 2017, Winer *et al.* used SEFC to detect blood cells flowing in the capillaries of human lips. By measuring the reflected light intensity of different cells, the team achieved imaging of RBCs and WBCs at different image depths *in vivo*, as well as differentiation of WBCs.[37] In 2019, Joseph *et al.* used a 796 nm adaptive optical fundus imaging system to measure the functional parameters and image blood cells in retinal vessels.[47] In 2020, McKay *et al.* used an oblique back-illumination capillary (OBC) microscope and 527 nm LED green light for the simultaneous high-resolution imaging of phase and absorption, and demonstrated label-free imaging of individual RBCs, WBCs, and platelets in the human tongue.[48]

Blood cell detection and classification results have become an important reference for modern medical diagnosis of patients' health status. In the past, different types of cells were counted manually, which understandably had low detection efficiency and unstable accuracy. In recent years, machine learning has been exploited for various applications in the biomedical and clinical fields.[49–53] In FC, it is used to improve the robustness and accuracy of cell counting and classification *in vivo*.[42,51,54] For example, in 2020, Kim *et al.* used deep learning to segment the nail fold capillary, and a WBC event detection algorithm to automatically count the WBCs. Deep learning can separate the capillaries missed by the traditional segmentation method, and the result is almost the same as that of manual segmentation, which improves the performance of counting.[55,56] Machine learning also facilitates noninvasive biopsies for tumor diagnosis. In 2021, Li *et al.* presented a deep learning-based framework to rapidly transform the *in-vivo* reflectance confocal microscopy (RCM) images of unstained skin into virtually stained hematoxylin and eosin-like images, and to rapidly perform virtual histology of *in-vivo*, label-free RCM images of basal cell carcinoma and melanocytic nevi with pigmented melanocytes.[57]

Table 1. Living cell classification via label-free IVFC.

Method	Simple model	Cell type	Reference
Endogenous absorption based on cells	Mouse ear	Melanocytes and RBC	45
Based on auto-fluorescence	Hamster pouch	RBC, WBC	46
	Zebra fish	RBC, WBC	39
Based on different in spectral characteristics	Human lips	RBC, WBC	37
	Retina of mice	Blood cells	47
	Human tongue	RBC, WBC, and platelets	48
U-Net network	Human nail fold	WBC	55
GAN network	Skin	tumor cell	57

4. Application of Label-free Method

Melanoma is the most serious of the three main types of skin cancer, and its incidence has increased significantly in fair-skinned people. The pigment in melanoma cells is a very good endogenous contrast agent and well suited for detection with PAFC. Some researchers (Zharov *et al.*, Liu *et al.*, and Niu *et al.*) performed a series of detections on circulating melanoma cells in a mouse model,[31,58,59] and confirmed that the PAFC technique can be used for *in-vivo*, label-free, and noninvasive detection. Extensive research has been conducted on melanoma in the human body.[60] Attia *et al.* and Hai *et al.* realized the measurement of the depth and volume of melanoma through photoacoustic tomography (PAT),[61,62] which can provide high contrast and detection ability for melanoma imaging. In 2019, Galanzha *et al.* used PAFC based on the cytophone technology to directly detect melanin CTCs in 1–2 mm deep hand veins of patients, which confirmed the clinical potential of PAFC.[63]

Circulating blood clots (CBCs) is a serious clinical complication in many medical procedures and diseases. Galanzha *et al.* could detect CBCs only in the superficial blood vessels of mouse models by using a custom-developed *in-vivo* PAFC platform.[64] Further, in 2018, the team combined an unfocused ultrasonic transducer with their initial platform to detect both red and white CBCs induced by microsurgery (such as needle or catheter insertion) and stroke in relatively large and deep blood vessels in rat and rabbit models. This technique is helpful in timely monitoring and treatment of CBCs.[65]

The quantification of WBC content and subtypes in blood is a routine clinical test. There are many methods for label-free imaging of WBCs *in vivo*, including photothermal, PAFC,[66] spectrally encoded detection, stimulated Raman scattering microscopy,[67] multiphoton microscopy, and oblique back-illumination capillaroscopy. These methods enable the detection, quantification, and determination of the WBC subtypes in the human skin dermis and dermal capillaries. In 2019, Pablo-Trinidad *et al.* used noninvasive optical systems and convolutional networks to detect severe neutropenia with high accuracy in 44 patients with absolute neutrophil counts of various levels.[68]

Many studies have shown that the lipid metabolism of tumors can be used as a marker for cancer detection.[69] In 2021, Andreana *et al.* used a hyperspectral coherent anti-Stokes Raman scattering (CARS) microscope to track the lipid content during the development of a zebrafish model, revealing the increase in lipid in tumor-developing zebra fish larvae.[70] (The application of label-free method is shown in Table 2).

Table 2. Application of label-free method.

Type	Method	Reference
Melanoma	PAFC and photoacoustic tomography	63
Blood clotting	PAFC	65
WBC	Noninvasive optical systems and convolutional networks	68
Lipid	CARS microscope	70

5. Conclusions and Discussion

Both labeled IVFC and label-free IVFC have undergone rapid development and technological innovation in their respective fields. Fluorescent IVFC can provide specific markers and bright contrast. However, it is limited by the toxicity of the fluorescent dye and depth of detection, and the development of additional nanoprobes can be expected in the future.[71] Label-free IVFC is a new method of cell counting and classification developed in recent years. The method can be classified into two types: One relies on the endogenous properties of the cells, such as autofluorescence, to enhance the high contrast of light absorption. The other combines absorption imaging with image processing methods such as deep learning to achieve cell counting and classification. Label-free IVFC has three advantages over labeled IVFC. First, it is not limited by the development of nontoxic labels and has greater clinical potential. Second, it broadens the scope of application of IVFC, making it possible to convert *ex-vivo* cell assays into *in-vivo* cell assays, thus allowing more information on cell activity to be obtained. Third, label-free IVFC makes it possible to monitor cellular activity over long periods of time. However, it also has some limitations.

First, the blood flow volume for the detection is limited, because the blood flow velocity is limited by the scanning speed of the microscope, which still takes days to monitor the entire blood volume in humans. In the future, unlabeled full-field imaging methods can be used to achieve large-scale simultaneous imaging of multiple blood vessels. By

utilizing the advantage of deep learning in cell feature extraction, simultaneous counting of unlabeled multiple types of cells can be achieved. In addition, improvements in the ultrasonic transducer can enhance the lateral resolution of imaging in PAFC and ensure high imaging quality.

Second, the sensitivity of label-free IVFC detection decreases with depth. In the future, OCAs can be combined with label-free IVFC to reduce light loss and exceed the limit of the conventional detection depth.[72,73] It is also possible to utilize some techniques such as multispectral PA imaging and phase microscope to improve the cell contrast.

Third, the accuracy of cell counting and identification has limitations related to the endogenous properties of cells and machine learning models. Therefore, the applicability of the current label-free FC remains limited.

Acknowledgments

This work was supported by the Key-Area Research and Development Program of Guangdong Province (2020B1111040001), the National Natural Science Foundation of China (62075042, 62205060, and 61805038), and the Research Fund of Guangdong-Hong Kong-Macao Joint Laboratory for Intelligent Micro-Nano Optoelectronic Technology (2020B1212030010). Special Fund for Science and Technology Innovation Cultivation of Guangdong University Students (No. pdjh2022b0543).

Conflicts of Interest

The authors have no conflicts of interest relevant to this article.

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