Multicolor multiphoton in vivo imaging flow cytometry

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Abstract: In vivo flow cytometry provides a non-invasive way of probing the biology of circulating cells during disease progression and studying cellular response to therapy. However, current methods provide little morphological information which potentially could be new biological marker for early disease diagnosis, and fail to reveal intercellular interactions. Here we report a multi-color, multiphoton in vivo imaging flow cytometry, to image circulating cells within the vasculature of scattering tissues at high spatiotemporal resolution. We apply it in imaging of cellular dynamics in bone marrow through the intact mouse skull, in situ deformability cytometry, distinguishing cellular clusters, and simultaneously monitoring multiple types of trafficking cells based on their morphologies and fluorescence emission colors.

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1. Introduction

Traditional flow cytometry (FC) is used routinely to acquire quantitative information about specific cell populations at high throughput, and has found extensive clinical applications in diagnostic pathology [1, 2]. However, blood extraction in conventional FC is invasive and the extensive processing required by in vitro detection can introduce potential artifacts [3]. Moreover, blood sampling reduces the sensitivity of capturing rare cells (such as circulating tumor cells) and prevents the long-term study of cell metabolism in their native complex biological environment [4]. In fact, detection and quantification of rare circulating cells in vivo are very important for early diagnosis of diseases (such as cancer, stroke, and inflammation), and long-term study is necessary for probing the biology of circulating cells during disease progression, and for studying cellular response to therapy (such as drugs and radiation) [1, 3–9]. To this end, several methods of in vivo FC have been developed to continuously monitor circulating cells in live animals without affecting the physiology of the subject, and found applications in many biomedical studies, such as cancer, immunology, and stem cells [3–5, 10–12]. A drawback of in vivo FC is that it generally provides little morphological information (such as the size, shape, morphology, and deformability of circulating cells), which can potentially be new biological marker that is sensitive to early disease development [13, 14].

The recent advent of in vivo imaging FC [8, 15], or flow cytography [14], enables researchers to record morphologies and intercellular interactions of trafficking cells in situ [1, 16]. Wide-field imaging based FC has been demonstrated to distinguish potential cellular clusters [15], and to assess cellular morphology and deformability in the native biological environment [8]. To get depth-resolved images, a three-dimensional (3D) image cytometer based on widefield structured light microscopy and high-speed remote depth scanning was developed, with 3D resolution to improve quantification of cytometric features [13]. However, wide-field imaging based methods are highly susceptible to optical scattering in biological tissue, and are limited to the superficial surface or transparent samples [17]. For deep tissue imaging, multi-photon microscopies, such as two-photon fluorescence microscopy (TPM) [18], remain the methods of choice. In this regard, laser scanning (point scanning) multiphoton imaging FC is ideal [17, 19] due to the intrinsic advantages in the 3D high resolution sectioning, the improved signal from both ballistic and scattered fluorescence emission, and the deep penetration depth of the near infrared laser excitation. Unfortunately, the current imaging speed of TPM is generally too slow to capture in detail the morphology of fast flowing cells [20]. This is primarily a consequence of limited scanning speed, particularly in the axial direction. Generally it is necessary to adopt 1D line scanning, instead of 2D cross-sectional scanning, to ensure adequately fast sampling of vascular dynamics (Fig. 1), which however loses the spatial information in the axial dimension [10]. Meanwhile, methods based on optical coherence tomography [5] and photoacoustics [14] have been developed for in vivo imaging FC.

To improve the axial scanning speed of TPM, several methods have been proposed, such as remote focusing microscopy [21, 22] and random access microscopy [23, 24]. But none of them has shown the capability of high-speed cross-sectional imaging for imaging FC. Recently, we have integrated optical phase-locked ultrasound lens (OPLUL) with a standard TPM for microsecond scale axial scanning [25]. Here we improve the cross-sectional frame rate to 2 kHz, and demonstrate the first multicolor multiphoton in vivo imaging FC, to our

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best knowledge. We applied it in imaging cellular dynamics in bone marrow through the intact mouse skull, and also showed in situ deformability cytometry [Fig. 1(b)]. With the improved cross-sectional frame rate, we were able to distinguish clusters and resolve morphologies of rapidly flowing cells. We also demonstrated the capability to concurrently monitor multiple types of circulation cells based on fluorescence emission wavelengths.

2. Experimental setup

The multicolor multiphoton in vivo imaging FC system is based on the integration of the OPLUL into a standard TPM, as shown in Fig. 2(a). The two-photon excitation femtosecond (fs) beam (80 MHz, 935 nm, 140 fs, Chameleon, Coherent) and the reference continuous-wave (CW) beam at 785 nm, both horizontally polarized, were combined with the dichroic beam splitter (DBS₁). After going through the polarization beam splitter (PBS), quarter waveplate (QWP) and ultrasound lenses (UL, TAG lens 2.0, TAG Optics), the beams were imaged onto a mirror (M₁) by a pair of relay lens (RL₁ and RL₁'). The reflected beams from M₁ went through the UL and QWP again, and were reflected by the PBS as their polarizations were rotated by 90° after the second pass of the QWP. The second pair of relay lenses (RL₂ and RL₂') were used to image the beam onto the x-y galvo scanner. The beams were separated...
by DBS\textsubscript{2}, with the reflected CW beam entering the photodiode (PD) and the transmitted fs beam employed for multiphoton excitation. The CW beam was spatially filtered by an iris before entering the PD, whose signal was used as the feedback for the phase-locked loop (PLL, HF2LI-PLL, Zurich Instruments) to maintain stable phase and amplitude of UL resonance. These elements formed the OPLUL, which combined with a conventional TPM enabled volumetric imaging and FC.

We conjugated the oscillating wavefronts \cite{25, 26} generated by the OPLUL to the pupil plane of the objective (Nikon 16 × , 0.8 NA) for high-speed axial scanning \cite{25}. The lateral and axial resolutions were ~0.5-0.6 µm and 2.9-4.6 µm, respectively. We drove the UL at the 450 kHz resonance, and achieved microsecond scale axial scanning. With a galvo scanner for transversal scanning, we obtained the kHz scale cross-sectional frame rate maintaining high spatial resolution. As an example, in Fig. 2(b), we show the raw data acquired by cross-sectional scanning a 1 µm fluorescent bead, in which the reference signal from the PD was used to reconstruct the cross-sectional image [Fig. 2(c)]. We used a high-capacity field-programmable gate array to record, process and display the data stream in real time.

3. Experimental results

We flexibly operated the system in both volumetric imaging \((x, y, z, t)\) and cross-sectional imaging \((x, z, t)\) schemes for FC applications. All procedures involving mice were approved by the Animal Care and Use Committees of Purdue University, HHMI Janelia Research Campus and National Institute of Allergy and Infectious Diseases, National Institutes of Health.

![Fig. 3. (a) Volume view of the vasculature in the bone marrow of mouse skull (Visualization 1). The whole volume is shown in a\textsubscript{1} (volume size: 300 × 300 × 121 µm\textsuperscript{3}), and the lower half of the volume is shown in a\textsubscript{2}. Blue: second harmonic generation signal of the bone matrix, red: Q-dot 655 labeled blood plasma, green: GFP-expressing cells. (b) Snapshots of a GFP-expressing cell (shown as the brown sphere) flowing through the vessel in the bone marrow, imaged at 7 Hz (Visualization 2). Volume size: 210 × 105 × 40 µm\textsuperscript{3}, \(t_1 = t_0 + 4.7\) s, \(t_2 = t_0 + 31.1\) s, \(t_3 = t_0 + 18.4\) s, \(t_4 = t_0 + 0.14\) s, \(t_5 = t_0 + 0.14\) s, \(t_6\) corresponds to Stack 3460 in Visualization 2. Dash line: the trafficking trace of the cell. (c) Centroids (circle dots) and long axes of the cell (curves of same colors with the corresponding centroids) highlighted in the red dot-dash circle in (b) (time separation: 14.3 s), from Stacks 3600, 3700, 3800, 3900, 4000, 4100, and 4200 in Visualization 2. We shifted the trace of the cell’s centroid to the red dot-dash line to show the time sequence.]

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3.1 In vivo volumetric imaging of cellular dynamics in the bone marrow through intact mouse skull

To show the advantage of our system in imaging through scattering tissues, we demonstrated the imaging of cellular dynamics in bone marrow through the intact mouse skull. The bone marrow is an important site, where all blood cells are formed and hematologic malignancies such as leukemia and metastasis of solid tumors emerge [27]. Noninvasive methods to image the cellular dynamics in the bone marrow are particularly important for stem cell studies, where any perturbation would disturb the fundamental biology [27, 28].

We used the CX3CR1<sup>gfp/gfp</sup> transgenic mice with EGFP-expressing monocytes, dendritic cells, natural killer cells, and brain microglia [29]. After removing the scalp, we directly imaged the dynamics of EGFP-expressing cells in bone marrow through the intact skull. The thickness of the skull above the bone marrow cavity was ~50 µm based on the second harmonic generation signal of the bone matrix [Fig. 3(a), and Visualization 1]. We recorded cellular trafficking and migrations inside the bone marrow [Figs. 3(b) and 3(c), and Visualization 2] at 7 Hz (210 × 105 × 40 µm<sup>3</sup>, 50-90 µm under surface of the skull, laser power: 70 mW). In Fig. 3(b), we show a cell trafficking through the vascular while simultaneously interacting with the microenvironment (from time point t<sub>1</sub> to t<sub>3</sub>). In Fig. 3(c), we show the traces of a migrating cell. This is the first report on in vivo fast volumetric (4D) imaging of cellular dynamics in the bone marrow without thinning the skull.

3.2 In situ deformability cytometry and imaging of intercellular interactions

Fig. 4. (a) Snapshots of a neutrophil flowing through the vascular junction, recorded at 28 Hz. Volume size: 151 × 28 × 40 µm<sup>3</sup>. Magenta: SR101 stained brain tissue, green: neutrophils. The volumes are from Stack 854-861 in Visualization 3. (b) Snapshots of a neutrophil crawling along the vessel wall. The neutrophil went into the circulation finally, as shown in Fig. (b). The volumes are from Stack 418, 419, 420, 429, 439, and 449 in Visualization 3. Same imaging parameters as in (a).

We have also applied our system as an in situ deformability cytometer for mechanical phenotyping. Cell deformability indicates changes in the cytoskeleton and nuclear organization, which may provide a label-free biomarker for determining cell states or properties [8, 30, 31]. Current techniques are mostly based on microfluidic devices for in vitro quantification [30, 31], which however may bring artifacts during the invasive sample isolations. In the native biological environment, circulating cells can undergo apparent deformations in geometrically irregular regions of the vasculature, such as bifurcations, curves, and narrowed areas [8]. Taking advantage of our continuous volumetric imaging system, we were able to quantify the deformability of circulating cells in situ. We used the Lyz<sub>2</sub><sup>gfp/+</sup> B6.Albino mice with GFP-expressing neutrophils [32]. After craniotomy and the installation of optical windows, we stained the brain tissue with sulforhodamine 101 (SR 101) for 2 minutes. We induced a gentle laser ablation of the neocortex to activate neutrophils and to recruit neutrophils from the bone marrow to the damage site through circulation system. We imaged the vasculature around the damage site (151 × 28 × 40 µm<sup>3</sup>, 10-50 µm under...
dura, 63 mW), and took snapshots of neutrophils when they passed through a bifurcation of blood vessels at 28 Hz (Fig. 4, and Visualization 3). From the volumetric imaging data, we were able to quantify the flowing speeds, which were 0.79 ± 0.42 (mean ± standard deviation) and 0.38 ± 0.15 mm/s in the main branch and one side branch, respectively. The velocity difference may indicate the physical pressure change that would result in deformation of the neutrophils passing by [Fig. 4(a)]. At the same time, some neutrophils were still able to crawl along the wall of the blood vessels at the bifurcation [Fig. 4(b)]. The capability of quantifying cell deformability and cell-vessel wall interactions may benefit early disease diagnosis and drug screening.

3.3 In vivo imaging FC for distinguishing cell clusters at high spatiotemporal resolution

Using a galvo scanner for transversal scanning, we achieved a cross-sectional (x-z) frame rate up to 2 kHz, which enabled us to distinguish cell clusters at high spatiotemporal resolutions. It was reported that although rare in circulation, clusters of the circulating tumor cells have much higher metastatic potential compared with single ones [33]. Quantifying the clusters of circulating cells and tracking their fates are critical in studying disease progression [15]. We mimicked this process by monitoring activated neutrophils after tissue damage. We used the DsRed$^{+/-}$ Lyz2$^{gfp/+}$ B6.Albino mice with GFP-expressing neutrophils. After inducing mild tissue damage in the ear pinnae, we imaged the trafficking of GFP-expressing neutrophils in the vasculature of the mouse ear. We took both volumetric imaging and cross-sectional imaging, along and across the blood vessel, respectively. With volumetric imaging (31 Hz,
168 × 28 × 40 µm³, 70-110 µm under surface, 72 mW), we were able to capture the clusters [Fig. 5(a), and Visualization 4] and calculate the flowing speed, while cross-sectional imaging (2 kHz, x × z: 28 × 40 µm², 70-110 µm under surface, 72 mW) provided cellular images with smaller distortions [inset in Fig. 5(b), and Visualization 5]. In the ~40 minutes of continuous cross-sectional imaging, no tissue damage was observed, and only ~10 out of 764 neutrophils were potential clusters. We were able to statistically analyze the temporal separation between consecutively passing neutrophils [Fig. 5(b)], and capture the morphology details of the clusters flowing at 0.82 ± 0.16 mm/s.

3.4 In vivo imaging FC for distinguishing cells based on morphologies and colors

Simultaneously quantifying multiple populations of circulating cells is important for disease diagnoses [34, 35]. For example, the ratio of neutrophil to lymphocyte is found to be a predictor of stroke [36]. Taking advantages of the high spatial resolution intrinsic to multiphoton point-scanning and the 2 kHz cross-sectional frame rate of our system, we were able to distinguish different cell types based on cellular morphologies. We collected mito-Dendra2 expressing lymphocytes, whose mitochondria are labeled with green-emitting dendra2, from the spleen of PhAMcre;transgenic mice, and transferred ~100 µL of the diluted cellular solution into the circulation system of Lyz2eGFP B6.Albino mice via tail-vein injections. After inducing mild tissue damage in the ear pinnae as described above, we imaged trafficking cells (flowing at 1.35 ± 0.16 mm/s) in a blood vessel of mouse ear with 22.4 × 40 µm² cross-sectional area at 2 kHz (60-100 µm under surface, 72 mW). In Figs. 6(a)–6(c) and Visualization 6, Visualization 7, and Visualization 8, we show the morphologies of the Dendra2-expressing lymphocytes and the GFP-expressing neutrophils. Although they were of the same emission color, we were able to distinguish these two cell types from their morphologies, and quantify their numbers and temporal distributions [Fig. 6(d)].

Fig. 6. (a–c) Cell morphologies (ObliqueSlice view in Amira) are distinguishable at high temporal and spatial resolutions. A potential cluster is shown in (b). Slices of three cross-sectional imaging sections (Visualization 6, Visualization 7, and Visualization 8) are shown in (a), (b), and (c). They were at 20 µm, 24 µm and 20 µm depth of the imaging volumes, respectively. Size: 22.4 × 40 µm² recorded in 20 ms at 2 kHz cross-sectional frame rate. (d) Temporal distribution of the lymphocytes with dendra2 expression in mitochondria and GFP-expressing neutrophils trafficking through the cross-sectional imaging plane.
Fig. 7. (a) Multicolor *in vivo* imaging of circulating cells. Size: 42 × 40 µm$^2$ recorded in 75 ms at 2 kHz cross-sectional frame rate. Magenta: SR101 stained blood cells, green: neutrophils. (b) Closely spaced neutrophil and SR101 stained blood cell. Size: 42 × 40 µm$^2$ recorded in 20 ms at 2 kHz cross-sectional frame rate. (c) Temporal distribution of neutrophils and SR101 stained blood cells trafficking through the cross-sectional imaging plane. Typical result is shown in Visualization 9 (timestamps are in ms).

Moreover, based on the broad multiphoton excitation spectra, we were able to quantify different types of circulating cells with the color-labeling strategy [34]. We injected ~100 µL SR 101 (10 mg/ml concentration in saline, 20 mg/kg) into the Lyz2$^{GFP^+}$ B6.Albino mice via tail-veins. After ~1 hour, the SR 101 in the plasma was cleared, and certain types of blood cells were stained. We introduced tissue damage in the mouse neocortex, and imaged cell trafficking (at 0.36 ± 0.03 mm/s) in the brain vasculature through cranial windows. We were able to capture the morphologies of the fast flowing SR101 stained cells and the GFP-expressing neutrophils at 2 kHz cross-sectional frame rate (42 × 40 µm$^2$, 10-50 µm under dura, 44 mW), even when they were close in time [Figs. 7(a) and 7(b), and Visualization 9]. We show their temporal distribution in Fig. 7(c).

4. Discussion

Different from the axial scanning methods based on translating objectives in which the vibration of the objective may transmit to the biological specimens, our method achieved microsecond scale axial scanning without introducing such perturbations. With the 2 kHz cross-sectional frame-rate, our system can flexibly perform volumetric imaging and cross-sectional imaging. Volumetric measurement enables deformability cytometry, imaging intercellular interactions, and qualifying flow speed. Cross-sectional measurement enables imaging cytometry. The integration with TPM allowed us to image vasculature in deep tissue at high resolution. Moreover, the broad multiphoton excitation spectra makes it possible to monitor multiple circulating cells of different emission colors.

In addition to TPM, our microsecond scale axial scanning method can also be combined with other multiphoton microscopies, such as coherent Raman microscopy [37] and stimulated emission microscopy [38], for lable-free imaging of circulation cells. This would be of great interest for both biological and clinical studies.

Integrating adaptive optics with our system, we can compensate the focus distortions induced by the inhomegenous refractive index distribution and increase the imaging depth.
[39–44]. Using a polygon scanner for transversal scanning (x scan), we can potentially improve the cross-sectional frame rate to 30 kHz or even higher value [45].

5. Conclusions

In summary, our multicolor multiphoton in vivo imaging FC system has superior performance in both volumetric imaging for studying cellular deformability/intercellular interactions and quantifying flow speed, and in cross-sectional imaging for capturing precise morphology information of fast circulating cells and distinguishing clusters and cell types. We expect its broad applications in biology and pharmacology.

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