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***In vivo* flow cytometer for real-time detection and quantification of circulating cells**

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Abstract

An *in vivo* flow cytometer is developed that allows the real-time detection and quantification of circulating fluorescently labeled cells in live animals. A signal from a cell population of interest is recorded as the cells pass through a slit of light focused across a blood vessel. Confocal detection of the excited fluorescence allows continuous monitoring of labeled cells in the upper layers of scattering tissue, such as the skin. The device is used to characterize the *in vivo* kinetics of red and white blood cells circulating in the vasculature of the mouse ear. Potential applications in biology and medicine are discussed.

Current methods to detect and quantify various types of cells within the blood stream involve extraction of blood from the patient or animal followed by *ex vivo* labeling and detection. For example, standard flow cytometry involves taking blood samples, fluorescently labeling specific cell populations, and passing these cells in a single file through a flow stream.¹ The cells are interrogated by a light source (usually a laser) to determine the types and number of cells based on their fluorescence and light-scattering signals. Another example is a hemocytometer, which involves manual counting of cells against a grid while viewing them with a microscope. Although both methods are useful, they provide only a single time sample. Consequently, if the cell population of interest varies unpredictably or rapidly with time, it is difficult to obtain a valid temporal population profile, since it is difficult to know when to sample. In addition, with both methods, blood must be withdrawn for each time point, and there is a significant time delay between blood withdrawal and analysis. The development of confocal and two-photon imaging techniques has allowed the detection of static and circulating fluorescently labeled cells *in vivo*.² However, extraction of quantitative information about the number and flow characteristics of a specific cell population can be extremely tedious. In addition, the high velocity of flowing cells, especially in the arterial circulation, makes it difficult and sometimes impossible to track the cells, even when images are captured at video rates. To remedy these problems, we have constructed a flow cytometer with the capability of detecting and quantifying the number and flow characteristics of fluorescently labeled cells *in vivo* and over a continuous time period.

The underlying principle of operation of the *in vivo* flow cytometer is confocal excitation and detection of fluorescently labeled cells in circulation. A schematic of the experimental setup is shown in Fig. 1. The animal to be studied is anesthetized and placed on the stage with its ear adhered to a microscope slide with glycerine. A blood vessel of appropriate diameter is identified (see below). Light from a He–Ne laser is then focused into a slit by a cylindrical lens and imaged across the selected blood vessel with a microscope objective lens (40×, 0.6

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numerical aperture). Red and infrared light sources are ideal for this purpose, because they provide good penetration through tissue and blood. The size of the slit at the focal plane of the sample is approximately $5\ \mu\text{m} \times 72\ \mu\text{m}$. The depth of focus (i.e., the FWHM of the light slit onto the sample in the axial direction) is approximately $50\ \mu\text{m}$, a value chosen to match the vessels of interest. The sample is positioned so that the long dimension of the slit traverses the width of the blood vessel; thus fluorescence is excited as the labeled cells in circulation pass through the slit. The emitted fluorescence is collected by the microscope objective, directed through the dichroic beam splitter (BS1), reflected by a mirror and a second dichroic beam splitter (BS2), and imaged onto a $200\ \mu\text{m} \times 3000\ \mu\text{m}$ mechanical slit, which is confocal with the excitation slit. This confocal arrangement eliminates light from out-of-focus fluorescent and scattering sources. Fluorescence is detected with a photo-multiplier tube placed directly behind the mechanical slit, sampled at a rate of 100 kHz with a data acquisition card, and displayed and stored on a computer. A 675-nm ($\pm 25\ \text{nm}$) bandpass filter placed in front of the confocal slit prevents most of the backscattered excitation light from entering the detector. The power of the He–Ne laser at the blood vessel is approximately 600 μW .

To identify the blood vessel location for measurement, the mouse ear is transilluminated with a green LED and imaged by the microscope objective lens onto a CCD camera. The green LED (520 nm) provides good contrast for blood vessels due to hemoglobin absorption. A dichroic beam splitter (BS1) is used to reflect the transmitted green light toward the CCD. The field of view of the transillumination system is $800\ \mu\text{m} \times 1000\ \mu\text{m}$ with a lateral resolution of 1.47 μm per pixel. In the skin of the mouse ear the blood vessels are typically located at depths of 70–100 μm from the skin surface. The appropriate vessel size range is 20–50 μm . Capillary vessels smaller than 20 μm are not used since tumor cells and some white blood cells may not pass through them freely. Moreover, capillary vessels yield count rates that are too low for most cells of interest. The upper size limit is determined by the depth of focus (50 μm in the current system) of the excitation slit. A fraction ($\sim 7\%$) of the backscattered He–Ne probe light is also reflected by BS1 onto the CCD, allowing us to align the slit onto the blood vessel. Precise determination of measurement location is important for temporal studies, since the measurements have to be taken at the same location over time for valid comparison of the data.

The digitized signal is analyzed off-line with software developed on the Matlab platform. The time sequence is filtered with a moving average window to remove high frequency noise. Control measurements performed at the data acquisition location before any fluorescent labels are introduced in the blood stream are used to determine the noise statistics, and only fluorescent peaks that exceed the noise level are counted. The number of fluorescent peaks, along with the height and FWHM of each peak, are determined with algorithms developed in house.

Figure 2 depicts typical data traces acquired from the mouse ear vasculature. Peaks correspond to fluorescence from circulating human red blood cells (RBCs), which were isolated, labeled *ex vivo* with 0.1-mM DiD (a lipophilic dye that binds to cell membranes), and injected into the mouse's circulation through the tail vein. The *ex vivo* labeling procedure was used rather than direct injection of DiD into the mouse to limit the fraction of labeled RBCs to $<1\%$ and to avoid overlap of signal pulses. Data were acquired from an artery and the corresponding vein to assess the instrument's capability of detecting differences in the flow characteristics. Figure 3 shows the pulse-width distribution for cells detected in the two types of vessels. From the pulse widths the flow velocities can be calculated to be $\sim 3\ \text{mm/s}$ for the artery and $1\ \text{mm/s}$ for the vein, which is consistent with previous reports using optical Doppler tomography.³ The variation in peak height can be attributed to several causes, including the orientation and the depth at which the (nonspherical) RBC passes through the vessel and the cell-to-cell variability in staining intensity. The pulse-height statistics is a subject of continuing investigation.

Our capability of quantifying the number of fluorescently labeled circulating cells in a reproducible manner is demonstrated in Fig. 4A. Measurements were recorded over a 3-day period from the same artery of a mouse injected with DiD-labeled human RBCs, as described above. The mean and standard deviation of the number of cells per minute passing through the selected artery on a given day was calculated from three traces that were 2 min in duration each. The number of detected circulating RBCs remains constant, as expected.⁴ The kinetics of circulating *ex vivo* labeled RBCs can be contrasted with the kinetics of mouse white blood cells (WBCs) labeled *in vivo* with a fluorescently tagged antibody. Specifically, 20 μ g of rat antimouse CD45 monoclonal antibody labeled with Cy-Chrome was injected through the tail vein of a 6–8-week-old BALB/c mouse, which was anesthetized with a mixture of ketamine and xylazine (7:1 ratio). *In vivo* flow cytometry measurements were performed at 0.4, 1.4, 4.3, 8.3 and 25 h after the injection. When the antibody was introduced in the vasculature, it labeled the circulating WBCs, which expressed the CD45 antigen on their surface. The increase in the number of fluorescently labeled WBCs detected within the first 1.4 h represents the kinetics of antibody binding. The number then decreases rapidly by approximately 75% within the first 8 hr. This decrease is understandable since the circulation time of some WBC populations, such as neutrophils, is of the order of hours.⁵ In addition, WBCs can be eliminated by either lysis or phagocytosis as a result of antibody binding.⁶ The latter measurements also demonstrate one of the key advantages of this technique; the ability to sample repeatedly the same animal over short and long periods to acquire information about dynamic changes of interest.

In summary, we reported on the development of a new technique that combines the concepts of standard flow cytometry and confocal detection to allow acquisition of flow cytometric information *in vivo* without the need to extract a blood sample. We demonstrate that with this technique we can quantify the number of fluorescently labeled circulating cells in a reproducible manner. In addition, we can characterize the flow characteristics of these circulating cells by determining the FWHM of the detected fluorescence peaks. The ability to monitor circulating cells *in vivo* in a quantitative way offers a number of advantages. For example, we can observe the cell population of interest in its native environment, free of artifacts potentially introduced by cell isolation and processing procedures required to perform conventional flow cytometry measurement. Furthermore, we can follow the same cell population continuously and over long periods to examine the dynamic changes in the circulation of different types of cells. We are currently using this technique to measure the circulation lifetime of different tumor cells and to study the relationship between metastatic potential and circulation time. Ultimately we want to investigate the use of the circulating tumor cell count for monitoring antitumor treatment outcomes. In addition, we are using the *in vivo* flow cytometer to monitor leukocyte populations in the peripheral circulation in response to therapeutic manipulation such as antibody therapy. Following the kinetics of WBC depletion is important in tissue and organ transplantation and autoimmune diseases such as rheumatoid arthritis and AIDS. For potential human applications (oral mucosa, nailfold, and sclera are some possible measurement locations where superficial blood vessels can be found) the major limitation of our technique is the need to inject a fluorescent probe, which negates some advantage of the noninvasive optical detection. In addition, to detect a specific cell population, the fluorescent probe has to be linked to a targeting molecule, usually an antibody. The antibody labeling often leads to depletion of the target cells (Fig. 4B), a potential problem for monitoring immune cells but probably not a problem for tumor cells. Continuing probe development will be needed to make measurements in human patients a reality. In the current stage the *in vivo* flow cytometer is a powerful research tool that can provide new insights in studies of animal models of disease.

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Fig. 1.

Schematic of the *in vivo* flow cytometer experimental setup: L1, condenser lens; OL, microscope objective lens (40 $\times$, 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, photo-multiplier tube.

Fig. 2.
Representative traces of fluorescently labeled human RBCs flowing through A, an artery and B, a vein of the mouse ear. Traces were acquired after implementing a 50-point moving window averaging of the original data.

Fig. 3. Histograms representing the number of peaks with a specific FWHM representing circulating DiD-labeled RBCs per minute in an artery (black) and a vein (gray) of a mouse ear. Note the shift to higher FWHM values for the vein data, representing slow blood flow characteristics.

Fig. 4.

A, Number of human RBCs, labeled *ex vivo* and injected in the mouse circulation through the tail vein, flowing through a mouse ear artery remains constant for a period of 3 days, as expected. B, In contrast, the number of WBCs, labeled *in vivo* with a fluorescently tagged antibody, varies in a dynamic and rapid way from the time of antibody injection.