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Two-photon *in vivo* flow cytometry using a fiber probe

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Abstract

We have demonstrated the use of a double-clad fiber probe to conduct two-photon excited flow cytometry *in vitro* and *in vivo*. We conducted two-channel detection to measure fluorescence at two distinct wavelengths simultaneously. Because the scattering and absorption problems from whole blood were circumvented by the fiber probe, the detected signal strength from the cells were found to be similar in PBS and in whole blood. We achieved the same detection efficiency of the membrane-binding lipophilic dye DiD labeled cells in PBS and in whole blood. High detection efficiency of green fluorescent protein (GFP)-expressing cells in whole blood was demonstrated. DiD-labeled untransfected and GFP-transfected cells were injected into live mice and the circulation dynamics of the externally injected cells were monitored. The detection efficiency of GFP-expressing cells *in vivo* was consistent with that observed in whole blood.

Keywords

Two-photon fluorescence; fiber probe; double-clad fiber; *in vivo* flow cytometry; circulating tumor cell; cancer; metastasis; green fluorescent protein (GFP)

1. INTRODUCTION

Current research on cancer metastasis emphasizes the detection and quantification of various types of disseminating tumor cells (DTCs) or circulating tumor cells (CTCs) [1, 2]. It has been reported that the presence of CTCs in the blood stream correlates strongly with cancer metastasis and progression [3]. Therefore, there is great interest in developing techniques to detect the presence of cancer cells in the blood stream. Quantitative detection of CTCs can help clinicians to assess patients' responses to therapy and can aid in prognosis diagnosis [2, 4, 5]. Conventional flow cytometry is the standard technique to rapidly obtain quantitative information on a large number of cells. However, because of the stable hydrodynamic flow stream required to achieve quantitative measurements of cell information, it can only be performed *in vitro* or *ex vivo*. Blood samples need to be extracted from patients or animals, followed by *ex vivo* labeling and detection. The removal of cells from their native environment and the additional processing before the measurements can introduce potential

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artifacts. In addition, the extracted blood samples contain different cell populations each time, which cannot reflect the real situation in the living body. Consequently, it is desirable to enumerate the cells *in vivo* in real time to monitor the progress of the disease [5, 6].

To noninvasively detect circulating cells *in vivo*, several efforts have been made using microscope-based one-photon or multiphoton excitation schemes [5–8]. However, because of the scattering and absorption of biological tissue, only the peripheral blood vasculature can be accessed by these free-space detection techniques. The blood volume sampled by these techniques is always less than 0.5 $\mu\text{L}/\text{min}$ (consider a blood vessel of $\sim 50\ \mu\text{m}$ in diameter with an average flow velocity of 5 mm/s) [9]. The concentration of CTCs in the blood stream is less than 1 cell per milliliter at the early stage of metastasis. In order to sample a large volume of blood, long-term monitoring is necessary. However, for free-space monitoring techniques, in order to keep the focus of the laser beam on the blood vessel, the subject has to be immobilized or anesthetized, which limits the acquisition time. The sampled blood volume is thus too small to practically identify the rare CTCs in clinical applications. We propose to use a sensitive fiber-optic probe as a minimally invasive technique to access large blood vessels deep inside the body to increase the sampled blood volume.

We have previously developed a two-photon optical fiber fluorescence (TPOFF) probe based on a single-mode fiber, and have used the probe to quantify fluorescence in solid tumors in live mice on a real-time basis [10, 11]. Using a double-clad fiber (DCF) as the probe, we have demonstrated more than one order of magnitude increase in the detected two-photon excited fluorescence signals [12–14]. Here we demonstrate the use of this DCF-TPOFF probe to conduct two-wavelength-channel flow cytometry both *in vitro* and *in vivo*. In addition to the advantage that the fiber probe can access larger blood vessels to sample more blood volume, the scattering and absorption problems due to turbid blood and tissue are circumvented. We have achieved a high detection sensitivity of green fluorescent protein (GFP)-expressing cells in whole blood and *in vivo* due to the reduced scattering and absorption compared to what was achieved with free-space detection techniques. Single-cell detection is ensured because of the highly confined two-photon excitation area. This present technique provides a promising way of conducting long-term real time monitoring of circulating cells in animal models or in patients to advance the understanding of cancer biology or post-therapy response surveillance.

2. MATERIALS AND METHODS

2.1 Two-channel two-photon optical-fiber fluorescence probe setup

The experimental setup is shown in Figure 1. Pulses generated by a Ti:sapphire oscillator (Coherent Inc., Mira 900) were dispersion pre-compensated to optimize the intensity at output of the fiber. The fluorescence signal was collected back by the same fiber and the objective, and separated from the excitation beam by a dichroic mirror. The fluorescence signal was further separated into two wavelength channels by another dichroic mirror (cutoff 610 nm). The fluorescence signals were filtered by band-pass filters (channel 1: 510/50 nm, channel 2: 680/30 nm) before detection by photomultiplier tubes (PMT, Hamamatsu, H7422-40). The outputs from PMTs were labeled in a router and then processed in a time

correlated single photon counting (TCSPC) module (SPC630, Becker and Hickl, GmbH). The DCF used in this study had an inner core diameter of 6 μm with an NA of 0.15 and a 123- μm inner cladding diameter with an NA of 0.46. The length of the fiber was about 1 m.

2.2 *In vitro* studies

The cells used in this study were from the mouse sarcoma MCA-207 cell line. Untransfected cells and GFP-transfected cells were maintained in the facility of the Michigan Nanotechnology Institute for Medical and Biological Sciences at the University of Michigan. The stably GFP-transfected cells were labeled with the membrane-binding dye DiD (Invitrogen) to produce the dual-labeled GFP-DiD cells according to manufacturer's protocol. The cells were then washed by PBS and re-suspended in PBS or 50% bovine whole blood and 50% PBS mixture at a concentration of 1×10^6 cells per milliliter. The cells were flowed through transparent plastic micro tubes (Tygon). One end of the tube was fixed. The fiber probe was inserted into the fixed end and controlled by a translation stage. The cells were injected into the other end of the tube through a syringe needle, and the flow rate was controlled with a syringe pump (KDS-100, KDS scientific).

2.3 Animals for *in vivo* studies

Male specific-pathogen-free CD1 30–32 mice were purchased from Charles River Laboratories (F38989, Portage, Michigan) and housed in a pathogen-free animal facility at the University of Michigan Medical Center. The handling of the mice was in accordance with the regulations of the University's Committee on the Use and Care of Animals as well as with federal guidelines, including the principles of Laboratory Animal Care [15]. The mice were anesthetized by inhalation of isoflurane before and during the measurements. DiD-labeled, untransfected MCA-207 cells or DiD-labeled GFP-transfected MCA-207 cells were injected through different places to monitor the circulating and depletion dynamics of the cells. The mice were unconscious but alive throughout the measurements. The mice were sacrificed after the experiments.

3. RESULTS AND DISCUSSIONS

3.1 *In vitro* studies

Representative photon-counting traces of dual-channel detection are shown in Figure 2. The long-wavelength ("long") channel corresponds to the emission wavelength range of DiD (680/30 nm). The short-wavelength ("short") channel corresponds to the emission wavelength of GFP (510/50 nm). The stably GFP-transfected MCA-207 cells are dual-labeled with the membrane-binding dye DiD. Each peak in the photon counting traces represents an event in the corresponding channel. Figure 2(b) is the magnified view of the photon counting trace. The peaks in the two channels nicely line up indicating simultaneous detection of dual-labeled cells in the two wavelength-channels. The ability to count thousands of cells per minute has been confirmed. In addition, the ability to resolve cells at the single-cell level is demonstrated. For this particular measurement, the cells flowed through a 250- μm inner diameter plastic tube at a flow rate of 100 $\mu\text{L}/\text{minute}$. The estimated average flow speed in the tube is 8.4 mm/s. Assuming a cell diameter of 10 μm and the lateral diameter of the two-photon excitation region at the fiber tip of 5 μm , we can estimate

the detection rate from the cell concentration and the aspect ratio between the size of the tube and the fiber core. The expected number of detected DiD events per minute is about 1000, which agrees with the result of the experiment. It is reasonable to assume that all of the DiD-labeled cells passing through the excitation region were detected.

Figure 3 shows the average number of events detected when flowing dual-labeled GFP-DiD cells in PBS and in 50% whole blood. The cells flowed in a 500- μm inner diameter plastic tube. The larger inner diameter tube was used to diminish the chance of blood clogging. The detected numbers of DiD events in PBS and in blood are about the same and agree with the expected values calculated from the flow rate and the flow tube size. If we assume all the dual-labeled cells passing through the excitation volume at the fiber tip are detected in the DiD channel, 49.5% of the total detected cells are also detected in the GFP channel for cell suspensions in PBS. For every detected GFP event in the short channel, 96.2% of them has a corresponding DiD event in the long channel within a 10-ms interval. For the cell suspensions in 50% whole blood, 33.3% of the GFP-DiD cells are detected in the GFP channel, while 90.0% of the GFP events in the short channel are correlated with DiD events in the long channel. According to the data, for all the detected GFP events, more than 90% of the events have corresponding DiD events in the long channel. Therefore, the assumption that all of the cells passing through the probe volume at the fiber tip are detected in the DiD channel was valid.

In a further investigation of the peak energy (total photons in the peak) distribution of the detected events (Figure 4, 5), we found that the signal strength detected for cell suspensions in PBS and in blood are almost identical. For DiD events (Figure 4), the mean numbers of photons per peak (mean fluorescence) are 820.8 and 989.5 respectively, for cell suspensions in PBS and in blood. The higher counts in blood might be due to its higher viscosity, which can make the cells linger longer around the probe volume at the fiber tip. The peak energy distribution is not affected by the corpuscular nature of blood, which is in dramatic contrast to the objective lens-based free-space detection scheme. In free-space measurements, about one order of magnitude reduction in the detected event photon counts was observed when flowing cell suspensions in whole blood [16, 17]. This is due to the scattering and absorption by the components in whole blood, such as red blood cells. The problem is even worse for the detection of GFP-expressing cells, because of the strong absorption of hemoglobin at the emission wavelength of GFP, which is centered at about 510 nm [18]. Moreover, the autofluorescence of human blood also peaks at about 510 nm, which increase the difficulty of GFP-expressing cell detection in whole blood [16]. Despite the difficulty of detection in whole blood or *in vivo*, there is great interest in detecting GFP-expressing cells in the blood stream due to its importance in biomedical science [8, 18]. With a similar signal-to-noise ratio in the DiD channel as shown in Figure 2, the typical detection rate for GFP-expressing cells in the free-space detection scheme is below 5% [16, 17]. The combination of two-photon excitation and fiber-optic detection scheme circumvents the scattering and absorption problems of cell detection in whole blood. Because all the excitation and collection happen only at the closest proximity of the fiber core, the two-photon excited fluorescence (TPF) photons can be collected directly before going through multiple scattering as in free space detection schemes.

The peak energy distributions of the detected GFP events are shown in Figure 5. The peak energy distributions of cell suspensions in PBS and in blood are similar. The blood does not have an obvious effect on the detected signal strength as we have seen in the DiD events. The mean photon counts of the detected GFP events are 227.5 and 232.2, respectively, for cell suspensions in PBS and in 50% whole blood. The reduced number of events detected in the GFP channel for the cell suspensions in PBS can be simply explained by the smaller TPF signal of the GFP compared to DiD. However, we noticed that the number of GFP events detected in 50% whole blood was only about two-thirds of the events in PBS (Figure 3). The reduced detection rate in the GFP channel can be explained by the raised background photon counts from the autofluorescence of whole blood. The mean photon counts for the background of the blood was 14 photons with a standard deviation of about 4 counts. The detection threshold for the events was set to be the background photoncount plus three standard deviations, which was 26 photons for the blood. On the other hand, the mean photon count of the background for PBS was 2.5 photons, with a standard deviation of 1.6 photons. The peak detection threshold for the cell suspension in PBS was 9 photons. Figure 6 shows the peak maximum distribution of the detected GFP events in PBS and in blood. Because the peak detection threshold for cells flowing in blood was 26 photons, peaks with peak maxima less than 26 could not be identified. Therefore, the fraction of total peaks with peak maxima of less than 20 photons was zero and the fraction for a peak maximum of 20–30 was only half of that for a peak maximum of 30–40 photons. The fraction of the peaks with a peak maximum lower than 26 photons for cells flowing in PBSB was about 30% as shown in Figure 6, which can neatly account for the reduced detectable events when the cells were flowing in whole blood. Although the higher background of blood makes the detection of GFP cells in whole blood more difficult, however, we have achieved a detection sensitivity one order of magnitude higher than with free-space techniques due to the elimination of the scattering and absorption problems.

3.2 *In vivo* studies

The TPOFF probe was then applied to monitor circulating cells in live mice. For proof of principle, the probe was inserted into the left liver lobe of the mice for the *in vivo* measurements. The liver was chosen for the proof-of-principle experiment because of the high density of blood vessels and abundant blood flow in the liver. Before the injection of labeled cells, the background signals in both the short and long channels were recorded as the control. The control traces are shown in Figure 7(a). The background trace in the long-wavelength channel was flat; no photon counts higher than 20 were recorded during an acquisition time of about 3 minutes. The mean photon count was 2.7 with a standard deviation of 2 photons. However, in the short-wavelength channel, we noticed periodic spiking with a period of about 2–3 seconds. The periodicity was the same as the respiration rate of the mice. Therefore the periodic spiking was believed to be due to the breathing movement. The fluctuations in the short channel make the identification of GFP cells *in vivo* more difficult. There are many kinds of intrinsic fluophores in the blood or tissue with emission wavelengths that overlap the emission wavelength of GFP. Fortunately, because of the capability of conducting dual-channel detection under two-photon excitation, we can use the long channel as the reference to confirm the arrival of a cell in the probe volume, and then look for a coincident signal in the short channel. Using the long channel signal as a

reporter to tell us the existence of a cell at the probe volume makes it possible for us to do ratiometric measurements *in vivo*, even with the high background in the short channel.

To demonstrate the ability of using the TPOFF probe to conduct cytometry *in vivo*, 50–100 μL of DiD-labeled MCA-207 normal cells with a concentration of $50 \times 10^6 / \text{mL}$ were injected into the tail vein or spleen of the mouse. A representative time trace is shown in Figure 7 (b). The cells injected through the tail vein entered the circulation system immediately. However, because the externally injected cancer cells were not intrinsic to the mouse, they were quickly depleted from the circulation. Figure 8 is the observed depletion time course for tail vein injected cells. The depletion dynamics occur on a similar time scale to the depletion of similar cells as have been reported in the literature [6, 19].

3.3 Monitoring GFP-expressing cells *in vivo*

Dual-labeled GFP-DiD cells were injected into the mice through multiple locations. The average numbers of detected events per minute in the two channels are illustrated in Figure 9. The red bars represent the number of cells detected in the long-wavelength channel, and the green bars are the number of events detected in both channels. Because of the sizes of the organs, the injected volumes of the cell suspensions were different. We injected 100 μL into the tail vein, 50 μL into the right lobe of the liver, 50 μL into the spleen, and 30 μL into the heart. The values plotted in the figure are the numbers normalized by assuming the same injection volumes as in the tail vein injection case. The number of events detected for cells injected into the tail vein and heart are similar, as they both enter the circulating directly. The number of events detected for cells injected into the spleen is the fewest since the cells need to be absorbed before they can enter the circulation to reach the liver. In addition, the cells could be depleted or lodged in the organs before they can enter the circulation. The overall possibility to have identifiable events occur simultaneously in both channels was $27\% \pm 7\%$. This value is consistent with the *in vitro* measurements in whole blood.

4. CONCLUSIONS

To summarize, a fiber-optic based two-photon excitation flow cytometry system has been developed. A double-clad optical fiber is used as the probe to achieve signal collection efficiency comparable to an objective-lens. Use of a single fiber to excite and detect multiphoton fluorescence circumvents scattering and absorption problems. The corpuscular nature of whole blood has negligible effect on the detected signal strength. High detection efficiency of GFP-expressing cells in whole blood and *in vivo* is demonstrated. The ability to conduct multi-channel detection with multiphoton excitation makes it possible to detect GFP-expressing cells *in vivo* when a noisy autofluorescence background is present in the corresponding wavelength channel. The ability to efficiently detect GFP-expressing cells *in vivo* may advance the understanding of cancer metastasis in animal models. The capability to access large blood vessels makes this technique a practical solution for long-term real-time monitoring of rare circulating cells in disease diagnosis.

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Fig. 1.
Experimental setup.

Fig. 2.

(a) Representative two channel detection photon counting traces of dual-labeled GFP-DiD cells flowing in a 250- μ m tube. The blue line represents photons collected in the “long” channel (680/30 nm, corresponding to DiD emission wavelength) and the green line represents photons collected in the “short” channel (510/50 nm, GFP). **(b)** Magnified view of the dual-channel detection raw data. The upper panel shows the peaks detected in the short channel (GFP), and the lower panel shows the peaks detected in the long channel (DiD).

Fig. 3.

Number of cells detected per minute when flowing dual-labeled GFP-DiD cells in (a) PBS and in (b) blood. Control (yellow bars): events detected in the long-wavelength channel when flowing non-labeled untransfected MCA-207 cells. For the GFP-DiD cell suspension in PBS (a), the detected number of events in the GFP corresponding channel (green bar) is 49.5% of number of the total detected events in the DiD channel (blue bar), while 96.2% of the detected GFP cells have corresponding DiD events within a 10-ms interval (green bar with blue stripes). For the cell suspension in 50% whole blood (b), the detected number of GFP events are 33.3% of that of the DiD events while 90.0% of the events in the GFP channel have corresponding DiD events in the long channel.

Fig. 4. Distribution of the total photon counts in each peak detected in the DiD channel. (a) Cell suspensions in PBS. (b) Cell suspensions in 50% whole blood.

Fig. 5. Distribution of the total photon counts in each peak detected in the GFP channel. (a) Cell suspensions in PBS. (b) Cell suspensions in 50% whole blood. (Data were combined from different acquisition periods.)

Fig. 6.

Peak maximum distribution of GFP events for cells flowing in (a) blood and (b) PBS. The mean photon count for the background of blood was 14 photons with a standard deviation of about 4 counts. The event detection threshold for the peaks in blood was 26 photons. The mean photon count for the background of PBS was 2.5 photons, with a standard deviation of 1.6 photons. The peak detection threshold for the cell suspension in PBS was 9 photons.

Fig. 7.

(a) Control traces of two-channel two-photon intravital fiber-optic flow cytometry when inserting the fiber probe into the left liver lobe of a mouse without injecting labeled cells in the mouse. Each time point represents the total number of photons detected in 1 ms. The green line represents the short wavelength channel (corresponding to the GFP emission wavelength). The blue line represents the long wavelength channel (corresponding to the DiD emission wavelength). **(b)** Representative photon counting trace when 2.5×10^6 DiD-stained untransfected MCA-207 cells were injected into the spleen of the mouse.

Fig. 8.
Detected events time course in the long channel after injecting 100 μL of DiD-labeled MCA-207 cells at a concentration of $50 \times 10^6 / \text{mL}$ into the tail vein.

Fig. 9.

The number of detected events in the long channel (red bars, DiD) and in both channels (green bars, GFP-DiD) per minute in the *in vivo* measurements. The fiber probe was put on the left lobe of the liver and the dual-labeled cells were injected through different places: tail vein, right lobe of the liver, spleen, and the heart.