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Validation of a Device for Fluorescence Sensing of Rare Circulating Cells with Diffusive Light in an Optical Flow Phantom Model

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Abstract - **Detection and quantification of rare circulating cells in biological tissues is an important problem and has many applications in biomedical research. Current methods normally involve extraction of blood samples and counting of cells *ex vivo*, or the use of microscopy-based fluorescence *in vivo* flow cytometry. The goal of this work is to develop an instrument for non-invasively enumerating very rare circulating cells in small animals with diffuse light with several orders of magnitude sensitivity improvement versus current approaches. In this work, we describe the design of our system and show that single, fluorescent microspheres can be detected in limb-mimicking optical flow phantoms with varying optical properties chosen to simulate *in vivo* conditions. Further, we demonstrate single cell counting capabilities using fluorescently (Vybrant-DiD) labeled Jurkat and Multiple Myeloma cells. Ongoing work includes *in vivo* testing and characterization of our system in mice.**

I. INTRODUCTION

THERE is a great deal of interest in the development of technology for *in vivo* detection and quantification of rare circulating cells for preclinical applications.[1-4] Current methods for cell enumeration require the extraction of blood and the analysis of these samples *ex vivo* using methods such as hemocytometry and flow cytometry.[5] These approaches allow sampling of relatively very small fractions of the total circulating blood volume and may not be accurate indicators of actual circulating cell populations, especially if these populations vary with time.[2] Recently, microscopy based *in-vivo* flow cytometry has been developed to allow non-invasive detection of fluorescently labeled cells in small arterioles in the ear or retina of mice or rats.[1] While this technique is highly valuable, the limitation is that only small blood volumes can be monitored (about 1μL/min) and very rare cells may therefore escape detection entirely. The lower threshold limit for these instruments is on the order of 10^3-10^4 cells / mouse; the overall goal of this work is therefore to design an instrument that would allow for detection in the range of 1000 cells / mouse or less.

Our strategy was to examine larger blood vessels in the legs or tail of a mouse with diffuse light and full angle illumination and detection. In principle, this will allow monitoring of significantly larger blood volumes (~0.2-0.5mL/min) so that the entire blood volume of a small animal can be interrogated in minutes. If successful, our design could potentially yield several orders of magnitude sensitivity improvement over current approaches. We also note that the purpose of this work is to develop a system specifically for pre-clinical research in mice (as opposed to a human-scale clinical instrument) including the study of cancer metastasis at early stages and tracking of hematopoietic stem cells. Up-scaling of this approach for use in human subjects is therefore not of immediate interest but could potentially be addressed in the future.

In this presentation, we first describe our instrument and then demonstrate its operation with a limb-mimicking optical flow phantom model with circulating fluorescent microspheres and fluorescently labeled cells. As we discuss, one of the main foreseeable barriers in the effectiveness of this technique is fluorescent signal attenuation due to optical absorption from native chromophores and blood content in biological tissue. We describe a series of experiments designed to investigate the effects of attenuation from India Ink and whole blood and verify that the fluorescent signal from cell-simulating microspheres is clearly visible over a range of optical properties expected *in vivo*. We also demonstrate that the system is capable of detecting and accurately counting fluorescently-labeled single cells in the phantom with linear flow rates up to 15 cm/s and concentrations less than 100 cells/mL. Overall, the results shown here verify that our system is capable of detecting fluorescent microspheres and fluorescently labeled cells in phantoms with similar optical properties to that of mice limbs. Ongoing *in vivo* studies with circulating cells in nude mice will also be described.

II. METHODS AND MATERIALS

A. System Design

A schematic and photograph of our system is shown in Figure 1. The system consists of a 6-channel fiber-coupled detector ring and two modulatable 642nm (DL640-050-O,

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CrystaLaser, Reno, NV) excitation lasers. The output of each laser is passed through 640nm interference 'clean-up' filters (Z640/10x, Chroma Technology, Rockingham, VT) before illuminating the sample. For the experiments described here, the lasers were operated in continuous wave (CW) mode, but we plan to modulate them in the future to allow tomographic localization of fluorescent sources in the sample cross-section. Emitted fluorescent light is detected by an array of six optical fibers coupled to an eight channel photomultiplier

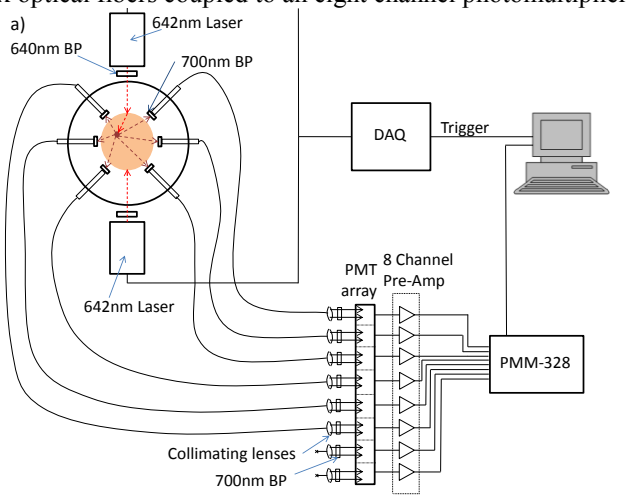

Figure 1. a) Instrument schematic, and b) A photo of the instrument showing the path of the excitation sources (red arrows) and an illuminated optical phantom in the center of the ring.

tube (PMT; H9530-01, Hamamatsu Photonics, Japan). Custom made 700nm interference filters with a 50nm bandpass (ET700/50, Chroma Technology) were placed on both ends of the detector fiber to block out source light as well as to minimize auto-fluorescence of the optical fibers. Detector fibers are terminated on custom designed lenses and filter mounts to ensure that the incident light is properly filtered before entering the PMT. The output from each PMT anode is then passed through a fast preamplifier (HFAM-26dB-10, Becker & Kickl, Berlin, Germany) and single photon pulses are counted with a multi-channel scalar board (PMM-328, Becker & Hickl) which independently detects very small changes in light intensity from the sample for each channel. For the experiments conducted here, the photon counting cards were configured to sample at a rate of 200 samples/sec. The data was then summed over 10 points to yield a final sampling rate of 20 samples/sec improving signal to noise ratio. As we discuss, with the low background and photon counting capabilities of our system, we were able to successfully detect single fluorescent microspheres and fluorescently labeled cells as they were passed through the limb mimicking optical phantom (section 2B) at physiological flow rates.

B. Optical Flow Phantom with Varying Absorption

We developed a set of limb-mimicking flow phantoms to have similar optical properties, background autofluorescence and linear flow speeds to that of a mouse limb or tail. These were fabricated from polyester resin and placed in a 3mm diameter by 1 cm length cylindrical mold with a length of Microbore Tygon tubing (TGY-010-C, Small Parts, Inc., Seattle, WA) passing through the center, which allows for the passage of microspheres and fluorescently labeled cells. The Tygon tubing was connected to a microsyringe pump (70-2209, Harvard Apparatus, Holliston, MA) allowing circulation of microsphere and cell suspensions at arbitrary flow rates in the range of 300 μm/s to 15 cm/s. For the experiments described here, we used a flow rate of 1 cm/sec, which is typical of what is expected *in vivo*.[6] The fluorescent microspheres used for these experiments were flow cytometer reference beads (6μm PeakFlow Claret, P-24670, Invitrogen, Carlsbad, CA) which were chosen to have similar size, fluorescence spectra and emission intensity to that of a cell labeled with Cy5.5 dye. These have an absorption peak at 645nm and an emission peak at 680nm.

The scattering and absorption optical properties of the phantom material could be varied by the addition of TiO_2 and India ink to the resin, respectively.[7] To understand the effects of optical properties of the detected signal from individual fluorescent microspheres, we fabricated two sets of optical phantoms (N=3 each); the first with reduced scattering coefficient μ_s'=15cm^{-1} and absorption coefficient μ_a=0.1cm^{-1} and the second with μ_s'=15cm^{-1} but significantly higher absorption μ_a=0.3cm^{-1}. These absorption coefficients were chosen to represent the predicted range at red and near-infrared wavelengths *in vivo*.[8] Microspheres were then passed through both sets of phantoms to investigate the relative change in fluorescence intensity (i.e. "spike height").

C. Varying Absorption of the Microsphere Suspension

To understand the potential effects of blood attenuation on the measured fluorescent signal, we varied the attenuation of the microsphere suspension media. Normally, microspheres were suspended in a Phosphate Buffered Saline solution (PBS) at concentrations of 1000 spheres/mL or less before they were passed through the phantoms. The effect of the absorption coefficient of this suspension was investigated first by the addition of (black) India ink to the PBS itself at increasing concentrations from μ_a = 0 to 0.6 cm^{-1} in step sizes of 0.15 cm^{-1}. As above, microspheres were passed through the flow phantom (μ_a = 0.1 cm^{-1} and μ'_s = 15cm^{-1}) at

a constant flow rate of 1cm/sec and the change in spike heights with increasing μ_a was noted.

Second, we increased the suspension attenuation in a more physiologically realistic way by the addition of whole blood to the PBS-microsphere suspension. This was done by increasing the whole blood to PBS ratio up to 1:2. The relative change in spike heights from the microspheres was analyzed with increasing ratios of blood to PBS.

D. Labeling and Fluorescent Detection of Jurkat and Multiple Myeloma cells

Finally, the system was tested with two different fluorescently labeled cell lines, specifically, Jurkat T-Lymphocyte cells and Multiple Myeloma (MM) cells. In each case, the cells were suspended in RPMI at a concentration of 1e6 cells/mL. Vybrant DiD cell labeling

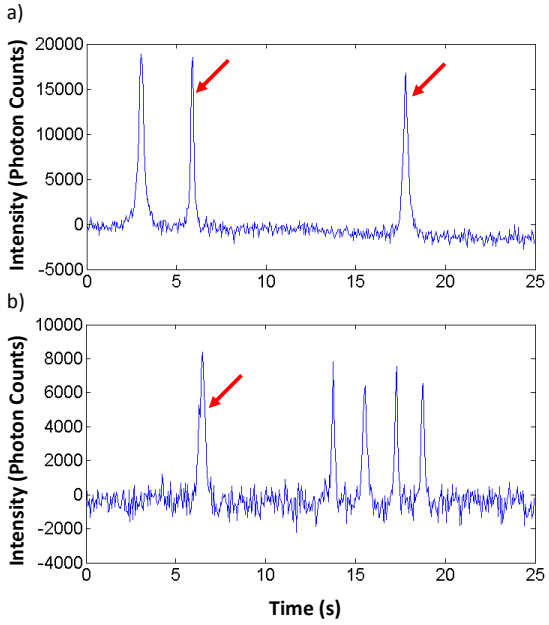

Figure 2. Fluorescent signals ('spikes', arrows) from single fluorescent microspheres as they pass through the instrument shown in figure 1. Flow phantom optical properties were a) μ'_s = 15cm^{-1} and μ_a=0.1cm^{-1}, and, b) phantom μ'_s = 15cm^{-1} and μ_a=0.3cm^{-1}. See text for details.

solution (V-22887, Invitrogen) with an absorption peak at 644nm and an emission peak at 665nm was added at a concentration of 1µM (MM cells) or 10µM (Jurkat cells) to the cell suspension and incubated for 30 minutes. The labeled cells were then resuspended in PBS and diluted down to 1000 cells/mL to be reasonably sure that single cells were passing through the field of view at any given time. For these experiments, the phantoms used had optical properties of μ_s'=15cm^{-1} and μ_a=0.1cm^{-1} and were running at a constant flow rate of 1cm/sec.

III. Results

A. Effect of Altering Phantom and Media Absorption

As shown in figure 2, when individual fluorescent microspheres flow through the instrument, transient fluorescent signals ("spikes") are clearly observable on each of the 6 detection channels. We have conducted significant studies to characterize our instrument (data not shown for brevity) and verified that our system is capable of detecting individual microspheres with a count accuracy of 90% or better at concentrations less than 1000 cells /mL and at linear flow speeds in the range of 300µm/s to 15 cm/s.

Two example data sets with fluorescent microspheres are shown, wherein phantoms with optical properties μ'_s=15cm^{-1} and μ_a=0.1cm^{-1} (fig 2a) and μ'_s=15cm^{-1} and μ_a=0.3cm^{-1} (fig 2b) were used. Here, background subtraction was applied which results in the small negative values observed in the data. It is evident from these data that while individual fluorescent spheres were observable in all cases investigated herein, as expected the absorption coefficient of the phantom had a significant impact on the detected fluorescence signal. From analysis of the total dataset, a mean reduction by a factor of 0.41 was observed in the amplitude of the fluorescent signals from each microsphere when the absorption coefficient was increased from 0.1 to 0.3cm^{-1}. The optical properties used here closely match stated literature values for optical properties at red and near infrared wavelengths; therefore, these data demonstrated the feasibility of this technique in vivo.

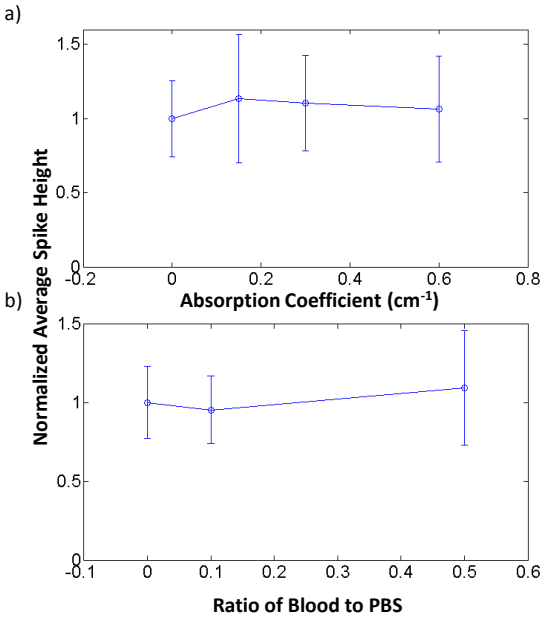

Figure 3. The normalized average microsphere fluorescent signal (i.e. "spike height") with a) increase in media μ_a from addition of India ink and b) increase in media μ_a by the addition of whole blood to the suspension in increasing ratios. The addition of absorbers to the media had negligible effect on the detected signal.

As noted above, one concern for the *in vivo* translation of this technique is the relatively high optical attenuation of whole blood. As shown in figure 3, we investigated this by adding either India ink (fig3a) or whole blood (fig3b) to the microsphere suspension. Here, the effect of the increased absorption of the media on fluorescent signal intensity (spike heights) is shown, normalized to the case of pure PBS media suspension. The error bars reflect the large variation in the spike heights observed for a particular sample run. Typically, this represents about 100 microspheres over 10 minutes. This

intra-experimental variability stems from the fact that although the total linear velocity is constant at 1cm/sec, the actual flow speed of the spheres across the flow profile vary significantly and lead to differences in the amount of time a spheres spends in the field of view, therefore altering the pulse width and height. This effect has been independently verified by fluorescence microscopic imaging of microspheres flowing through the tubing.

From these data, it can be seen that there is no significant decrease in average fluorescence intensity with increasing amounts of absorption in the PBS from either the India ink or whole blood. In summary, these experiments together imply that absorption of the blood will not be a limiting factor for our device when testing *in vivo*.

B. Detection of Jurkat and MM cells in Diffuse Phantom

Finally, figure 4 shows example data showing single Vybrant-DiD labeled Jurkat and MM cells through the flow phantom with optical properties of μ'_s=15cm^{-1} and μ_a=0.1cm^{-1}. As above, fluorescent spikes from individual cells were clearly visible as they pass through the instrument field of view. Cells were counted using a thresholding algorithm where a spike greater than one standard deviation above the background was counted as cells. A background measurement was taken prior to running cell measurements by running PBS through the phantom. Experiments to verify the quantitative accuracy of our system compared to conventional flow cytometry techniques are currently underway. In general, a small mean spike height and larger intra-spike variation was observed with cells compared to experiments with microspheres. This was due in part to the variations in flow velocity discussed earlier, but also due to the distribution of labeling efficiency of the cells which results in greater heterogeneity of the fluorescence signals. Further, the fluorescence signal measured with the two cell lines was similar. Therefore, both cell lines will be detectable over the range of optical properties used in section 3A above and by extension most likely in vivo. *In vivo* tests are currently underway and we will report on them in our presentation.

IV. Discussion

In summary, the results presented here demonstrate the feasibility of detecting cell-simulating fluorescent microspheres and fluorescently labeled cells in diffusive media with size, flow speeds and optical properties closely matching that of biological tissue. It was demonstrated that microspheres could be clearly detected while varying the absorption coefficient of the suspension over a wide range of values. We have also shown that it is possible to detect fluorescently-labeled Jurkat T-Lymphocyte and Multiple Myeloma cells in limb-mimicking flow phantoms, and that the fluorescence intensity is on the same order of magnitude as the fluorescent microspheres. In combination, these data illustrate the feasibility of our approach for detecting individual circulating cells in small animals *in vivo*.

We are now conducting preliminary *in vivo* studies in nude mice with circulating fluorescently-labeled MM cells. By interrogating larger blood vessels with higher (0.2-0.5 mL/min) blood flow rates, this instrument has the potential to allow monitoring of the entire blood volume of a mouse in minutes. This would allow for the detection of cells at lower concentrations on the order of 100 cells/mouse, allowing orders of magnitude increase in sensitivity over current *in vivo* flow cytometry approaches.

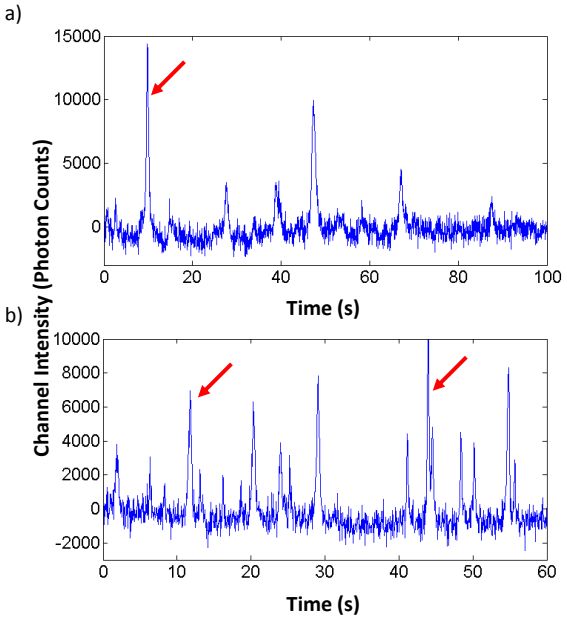

Figure 4. Example measurements from a) fluorescently labeled Jurkat T-Lymphocyte cells in a diffuse phantom and b) fluorescently labeled Multiple Myeloma cells in a diffuse phantom.

V. Acknowledgments

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References

[1] Alt, C., et al., "Retinal flow cytometer" Opt. Lett. 32(2), 3450-2 (2007).

[2] Novak, J., et al., "In vivo flow cytometer for real-time detection and quantification of circulating cells" Opt. Lett. 29(1) ,77-9 (2004).

[3] Georgakoudi, I., et al., "In vivo flow cytometry: a new method for enumerating circulating cancer cells" Cancer Res. 64(15), 5044-7 (2004).

[4] Boutrus, S., et al., "Portable two-color in vivo flow cytometer for real-time detection of fluorescently-labeled circulating cells" J. Biomed. Opt. 12(2), 020507 (2007).

[5] Shapiro, H.M., [Practical Flow Cytometry, 3rd Ed.], Wiley-Liss, New York, (1995).

[6] Wagner, S., et al., "Time-of-Flight Quantitative Measurements of Blood Flow in Mouse Hindlimbs" Journal of Magnetic Resonance Imaging 19, 468-474 (2004).

[7] Baeten, J., et al., "Development of fluorescent materials for Diffuse Fluorescence Tomography standards and phantoms" Opt. Express 15(14), 8681-94 (2007).

[8] Niedre, M., et al., "Time-resolved imaging of optical coefficients through murine chest cavities" J. Biomed. Opt. 11(6), 064017 (2006).