Signal and depth enhancement for in vivo flow cytometer measurement of ear skin by optical clearing agents

Yimin Ding,1,5 Jing Wang,2,5 Zhichao Fan,3,4 Dan Wei,1 Rui Shi,2 Qingming Luo,2 Dan Zhu,2,6 and Xunbin Wei1,3,4,*

1 State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, School of Biomedical Engineering, Shanghai Jiao Tong University, 1954 Huashan Road, Shanghai 200030, China
2 Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, 1037 Luoya Road, Wuhan 430074, China
3 Institutes of Biomedical Sciences, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China
4 Department of Chemistry, Fudan University, 220 Handan Road, Shanghai 200433, China
5 These authors contributed equally to this work.
6 dawnzh@mail.hust.edu.cn
7 xwei01@sjtu.edu.cn

Abstract: The in vivo flow cytometry (IVFC) has shown a great potential for detecting circulating tumor cells quantitatively in the bloodstream. However, the detection depth suffers from the strong light scattering of tissue. In this study, an innovative ear skin optical clearing agent (ESOCA) is employed to improve the signal quality of the IVFC. Our results show that compared with commonly used glycerol, topical application of ESOCA can enhance the transmittance of rat ear significantly in vivo. The labeled red blood cells can be detected by the IVFC with higher signal quality and greater detection depth. This study is very helpful for potential tumor metastasis studies by the IVFC in deep tissues.

©2013 Optical Society of America

OCIS codes: (170.1470) Blood or tissue constituent monitoring; (170.1530) Cell analysis; (170.3660) Light propagation in tissues;

References and links

1. G. S. Terentyuk, G. N. Maslyakova, L. V. Suleymanova, N. G. Khlebtsov, B. N. Khlebtsov, G. G. Akchurin, I. L. Maksimova, and V. V. Tuchin, “Laser-induced tissue hyperthermia mediated by gold nanoparticles: toward cancer phototherapy,” J. Biomed. Opt. 14(2), 021016 (2009).
2. W. Lu, Q. Huang, G. Ku, X. Wen, M. Zhou, D. Guzatov, P. Brecht, R. Su, A. Oraevsky, L. V. Wang, and C. Li, “Photoacoustic imaging of living mouse brain vasculature using hollow gold nanospheres,” Biomaterials 31(9), 2617–2626 (2010).
3. A. Herrman, M. Kortylewski, M. Kujawski, C. Zhang, K. Reckamp, B. Armstrong, L. Wang, C. Kowolik, J. Deng, R. Figlin, and H. Yu, “Targeting Stat3 in the myeloid compartment drastically improves the in vivo antitumor functions of adoptively transferred T cells,” Cancer Res. 70(19), 7455–7464 (2010).
4. I. J. Fidler, “The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited,” Nat. Rev. Cancer 3(6), 453–458 (2003).
5. I. Georgakoudi, N. Solban, J. Novak, W. L. Rice, X. Wei, T. Hasan, and C. P. Lin, “In vivo flow cytometry: a new method for enumerating circulating cancer cells,” Cancer Res. 64(15), 5044–5047 (2004).
6. J. Novak, “Development of in vivo flow cytometry,” Ph.D. dissertation, Dept. Mech. Eng., Harvard Univ., Cambridge, MA, 2004.
7. Z. C. Fan, J. Yan, G. D. Liu, X. Y. Tan, X. F. Weng, W. Z. Wu, J. Zhou, and X. B. Wei, “Real-time monitoring of rare circulating hepatocellular carcinoma cells in an orthotopic model by in vivo flow cytometry Assesses Resection On Metastasis,” Cancer Res. 72(10), 2683–2691 (2012).
8. J. Novak, I. Georgakoudi, X. Wei, A. Prossin, and C. P. Lin, “In vivo flow cytometer for real-time detection and quantification of circulating cells,” Opt. Lett. 29(1), 77–79 (2004).
9. J. Guo, Z. Fan, Z. Gu, and X. Wei, “Studying the role of macrophages in circulating prostate cancer cells by in vivo flow cytometry,” J. Innov. Opt. Health Sci. 5(4), 1250027 (2012).
10. Y. Li, J. Guo, C. Wang, Z. Fan, G. Liu, C. Wang, Z. Gu, D. Mosig, and X. Wei, “Circulation times of prostate cancer and hepatocellular carcinoma cells by in vivo flow cytometry,” Cytometry A 79(10), 848–854 (2011).
11. A. N. Bashkatov, E. A. Genina, and V. V. Tuchin, “Optical properties of skin, subcutaneous, and muscle tissues: a review,” J. Innov. Opt. Health Sci. 4(1), 9–38 (2011).
1. Introduction

The metastasis of tumor cells is a great threat to human life. The study of circulating tumor cells (CTCs) in the bloodstream has been a research focus. Optical techniques have been widely applied to study molecular mechanisms of tumor metastasis [1–3]. However, conventional optical methods for monitoring CTCs involve extraction of the blood from the patient or animal and counting radio-labeled or fluorescently labeled cells [4], which are invasive and difficult to record the dynamics of CTCs [5–7].

A recently developed optical technique, namely “in vivo flow cytometry” (IVFC), can overcome these restrictions. IVFC is based on the confocal excitation and detection of fluorescently labeled cells in circulation [8, 9]. It has the capability to count fluorescently labeled circulating cells in live animals, which can be applied to study the mechanism that governs the early steps in tumor cell spreading through the body [5–10].

However, like other optical methods, the detection depth of IVFC is limited, due to strong light scattering within tissues [11]. Tuchin et al. proposed the concept of optical clearing by immersion of tissues into optical clearing agents (OCAs), which can improve light penetration depth in tissues and enhance optical imaging depth [12–19]. Traditionally, glycerol is used in the IVFC experiments to provide slight adherence of the ear to the slide and facilitate the light transmission between the mouse ear and the slide, which also acts as an optical clearing agent [20]. It is noted that, due to the hydrophilic property of glycerol, its osmosis through the...
lipophilic stratum corneum is poor, which results in a minimal and extreme slow penetration of glycerol through intact skin [21–23].

Recently, we have developed an ear skin optical clearing agent (ESOCA), which can make the ear skin more transparent [24], and improve the contrast for laser speckle imaging. Here, we have investigated the efficacy of ESOCA for improving the IVFC detection. The signal intensity, peak number and detection depth of the IVFC are compared with those in the glycerol-treated group. The signal-to-noise ratio (SNR) is also calculated to evaluate the improvement in signal quality. We have demonstrated that after the application of ESOCA, fluorescence signals from deeper skin can be detected in the IVFC. In addition, both the signal intensity and SNR in deep skin are improved significantly.

2. Materials and methods

2.1 Chemical agents and animal preparations

The ESOCA used in this study was composed of several biocompatible agents [24], in which polyethylene glycol (PEG-400), fructose and thiazone were mixed with a volume ratio of 40:55:5. 100% glycerol was used as a control agent.

Animal care and experimental procedures complied with guidelines established by Shanghai Medical Experimental Animal Care Commission. Male Wistar rats (n = 6, 6-week old) for fluorescence detection were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. Male Wistar rats (n = 6, 6-week old) for evaluating optical clearing efficacy were purchased from Wuhan University Center for Animal Experiment (Wuhan, China). All of the animals were fed under specific pathogen free (SPF) level of feeding conditions. The study was approved by the Ethical Committee of Animal Experiments in School of Biomedical Engineering, Shanghai Jiao Tong University, and Huazhong University of Science and Technology. Rats were anesthetized with 1% pentobarbital sodium salt (0.01 mL/g rat weight). The hair on the ears was removed with depilatory cream (sensitive hair removal cream; M/s Veet).

The mouse red blood cells (RBCs) were isolated for fluorescence labeling. Suspensions of RBCs were incubated ex vivo at 37°C with 0.1 mM lipophilic carbocyanine DiD (1,1’-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine Perchlorate; Invitrogen) for 30 min, and then washed by phosphate-buffered saline for three times before the injection.

2.2 Optical fiber spectrometer for measuring the transmittance of rat ear

The transmittance of the rat ear was measured with an optical fiber spectrometer system (USB 4000, Ocean Optics, USA) [24]. The rat ear was fixed on a sample holder of a reflection and transmission stage (Stage-RLT-T, Ocean Optics, USA), which also had two fiber holders. A tungsten halogen light source (HL-2000-CAL, Ocean Optics, USA) coupled with an optical fiber (QP600-2-VIS/NIR, Ocean Optics, USA), which was fixed on the fiber holder, was applied to illuminate the rat ear. The other optical fiber connected to the spectrometer system was employed to collect the transmitted light. The spectral wavelength covers 400-900 nm. In this work, two collimating lenses were also used to measure the collimated transmittance, which were connected to two optical fibers.

First, the transmittance spectrum of the region of interest on the rat ear was measured. Then glycerol was topically applied on the ear skin for 10 min before the transmittance spectrum was measured again. Afterwards, the glycerol was gently removed from ear skin with filter paper. Then the saline solution was used to wash the residual glycerol. The transmittance spectrum of ear in the region of interest was measured again. Finally, the ESOCA was applied on the same area of dried ear skin for 10 min, before the transmittance spectrum was obtained once again. Before each measurement, optical clearing agents were removed to reduce the specular reflection.

To quantitatively evaluate the optical clearing efficacy induced by the two agents, the relative changes in transmittance was calculated as follows:

\[ \text{Relative Change} = \frac{\text{New Transmittance} - \text{Baseline Transmittance}}{\text{Baseline Transmittance}} \]

Received 22 Jul 2013; revised 4 Oct 2013; accepted 10 Oct 2013; published 17 Oct 2013

(C) 2013 OSA 1 November 2013 | Vol. 4, No. 11 | DOI:10.1364/BOE.4.002518 | BIOMEDICAL OPTICS EXPRESS 2520
\[
\Delta T = \frac{T_{treated} - T_{initial}}{T_{initial}}
\]  

where \( T \) denoted the transmittance, while the subscripts “initial” and “treated” denoted the ear before and after the treatment with optical clearing agents.

2.3 In vivo flow cytometry for detecting the fluorescently labeled cells

The in vivo flow cytometer was used to detect the fluorescence signal from DiD-labeled red blood cells in a confocal geometry (Fig. 1), based on previous groups’ experience [5–10, 25, 26]. Briefly, we used transillumination with a 535 ± 15 nm light emitting diode (LED) to visualize major veins and arteries in the mouse ear dermis. Typically, an artery of 50 to 70 \( \mu \)m in diameter was selected for data acquisition. A 635-nm laser was focused into a slit by a cylindrical lens and imaged across the selected ear artery. The size of the slit at the sample focal plane was ~5 × 72 \( \mu \)m. Fluorescently labeled cells were excited one by one as they flowed through the chosen artery, producing a burst of fluorescence for each cell. Fluorescence was detected with a photomultiplier tube placed directly behind the mechanical slit, and sampled at a rate of 5 kHz with a data acquisition card, which could be displayed and stored on a computer.

![Fig. 1. Schematic of the in vivo flow cytometer experimental setup: CL, cylindrical lens; MS: mechanical slit; AL1-AL3: achromatic lens; M: mirrors; BS1-BS2: dichroic beam splitters; objective lens (40 ×, NA = 0.6); F1-F2: band pass filters; PMT: photo-multiplier tube; DAQ: data acquisition.](image)

10\(^6\) DiD-labeled cells per 20 g body weight were injected through the tail vein. The chemical agents (100% glycerol or ESOCA) were topically applied on the surface of rat ear. The thickness of rat ear was measured as 384 ± 30 \( \mu \)m. The rat was then placed on a heated stage (37°C) while the ear side treated with agents was adhered onto the stage. Before experiments, a control measurement was performed to determine the noise level. Afterwards, an appropriate artery where the CCD image was sharpest was chosen for the IVFC measurement for 6 min while the depth of focus was recorded. As the focus went deeper, the IVFC measurement was repeated. When the depth reached a certain point, the CCD image was too blurry to find the vessel. Then, the whole ear was scanned deeper with a point-by-point manner. When the laser slit happened to come across a blood vessel, continuous cell
signal peaks could be seen from the screen, while a period of signal was recorded. This process continued until no signal could be found.

To quantify the number of labeled cells detected by the IVFC, a cell counting method was developed on the Matlab platform. The algorithm included two steps: moving average and adaptive peak-picking. In the first step, a period of digitized signal was filtered with a moving average window with the median of the target time sequence being selected as the baseline. Then the signal peak greater than the threshold was picked. The threshold was calculated according to previous work [27] using the following equation:

\[
\text{Threshold} = \text{Median} + \text{Multiplier} \times \frac{\text{MAD}}{0.6745}
\]  

(2)

where the MAD denoted the median absolute deviation of all the noise peaks, while the Multiplier was determined based on a control signal, usually set as 6 or 7. To further remove the noise signal, peaks with only single point were removed. Then the number of peaks could be determined.

In addition, the signal-to-noise (SNR) ratio was employed to evaluate the efficacy of optical clearing agents. It was defined as follows:

\[
\text{SNR} = \frac{A_s}{A_n}
\]  

(3)

where \(A_s\) represented the mean intensity of the signal peaks, while \(A_n\) represented the mean intensity of the noise.

2.4 Statistical analysis

Data were given as mean ± standard deviation. T-test was performed to determine significant difference between glycerol-treated and ESOCA-treated groups. The values were considered significantly different if \(p<0.05\).

Cumulative frequency analysis was performed to obtain insight into how often the full duration at half maximum was below a certain value.

3. Results

3.1 Improvement in transmittance of rat ear by ESOCA

Because the rat ear was thin, the collimated transmittance was measured here to evaluate the optical clearing efficacy. The transmittance showed a slight increase 10 min after topical treatment of glycerol on the skin of rat ear. The application of saline solution almost made the transmittance spectrum return to the initial state (Fig. 2(a)). Upon further treatment of ESOCA for 10 min, the transmittance increased significantly.

Fig. 2. (a) Representative transmittance spectra of rat ear before and after the treatment with glycerol, saline solution and ESOCA, respectively. (b) Relative transmittance spectra after the treatment of different agents.
To quantitatively evaluate the optical clearing efficacy induced by different agents, the mean value and deviation of transmittance from 12 ears (6 rats) at three wavelengths were calculated. The LED (535 ± 15 nm) was used for visualizing the blood vessels in the rat ear. 635 nm and 665 nm were the excited wavelength and the emitted wavelength for IVFC measurement. Table 1 summarized the transmittance (mean ± standard deviation) at 535 nm, 635 nm, and 665 nm before or after the treatment by glycerol, saline or ESOCA. The statistical analysis demonstrated that there were significant differences between the intact and ESOCA-treated skin (p<0.01) at the three wavelengths, but no differences between the intact and glycerol-treated or saline-washed skin.

| Treatment   | 535 nm (%) | 635 nm (%) | 665 nm (%) |
|-------------|------------|------------|------------|
| Intact      | 18.03 ± 2.93 | 31.44 ± 3.52 | 33.72 ± 3.99 |
| Glycerol for 10 min | 20.08 ± 3.13 | 34.62 ± 4.32 | 37.33 ± 4.37 |
| Saline washed | 19.04 ± 2.17 | 31.91 ± 4.64 | 34.01 ± 4.1  |
| ESOCA for 10 min | 35.59 ± 6.1** | 49.05 ± 4.81** | 52.07 ± 6.46** |

3.2 Changes in signal peaks of IVFC measurement after the application of OCAs

For in vivo flow cytometry, a typical signal peak represented one labeled cell passing the laser slit. With high peak intensity, the SNR was optimal for accurate cell counting. Therefore, peak intensity of IVFC signal was used to evaluate the optical clearing effect of ESOCA.

Fig. 3. IVFC signals of DiD-labeled red blood cells (left: treated by glycerol; right: treated by ESOCA). The signals were recorded at the depth of 80 μm (a), and 180 μm (b) for 6 minutes. Each peak represented a DiD-labeled cell traversing the excitation slit.
Within the depth of 80 μm in the skin, the fluorescence signal could be detected by the IVFC for both glycerol-treated and ESOCA-treated group (Fig. 3). Interestingly, the signal intensity in ESOCA-treated group was somewhat lower. When the depth increased to 180 μm, fluorescence signals were almost undetectable for glycerol-treated group, while the signals could still be well detected for ESOCA-treated group.

To quantitatively evaluate the optical clearing efficacy of skin in vivo, the average peak intensity was determined, as shown in Fig. 4(a). At a depth of 80 μm in the skin, the mean peak intensity decreased by 25.35% after topical application of ESOCA, compared to glycerol-treated group. The result from T-test indicated that there was a significant difference in signal intensity between these two groups. In contrast, at a deeper depth of 180 μm in the skin, the mean intensity increased by 31.04% after the application of ESOCA, which was also significantly different from that in glycerol-treated group.

For further comparison, the peak numbers and SNRs were calculated. The results were summarized in Fig. 4. Notably, the peak numbers (Fig. 4(b)) at superficial skin decreased after topical application of ESOCA, whereas the peak numbers at the deep skin was improved compared with that of glycerol group. The SNRs (Fig. 4(c)) at the superficial skin were very similar after either ESOCA treatment or glycerol treatment. However, in the deep skin regions, the SNRs for ESOCA-treated group were significantly better than those for glycerol-treated group, which demonstrated that ESOCA could enhance the signal quality effectively in the deep skin.

![Fig. 4. Comparison of signal peak properties after the treatment with glycerol or ESOCA, respectively (a.u.: arbitrary unit, p<0.05 *).](image)

3.3 Improvement in detection depth for IVFC measurement

Optical detection is generally limited by its measurement depth. In general, a confocal microscope can detect circulating cells within 100-150 μm in the skin. Currently, the IVFC is particularly useful for studying cancer metastasis in small animal model [10]. To apply the IVFC in deep tissue, it is essential to improve the detection depth of IVFC. The variations of peak intensity as a function of depth were shown in Fig. 5. Interestingly, we found out that the IVFC was able to detect signals from the deep tissue, even at depths difficult for fluorescence imaging. Specifically, after the treatment with ESOCA, the signal could still be detected by IVFC even at a depth of 330 μm which was significantly deeper than the counterpart detection depth of 250 μm after the application of glycerol. That was, compared with glycerol, the detection depth of IVFC was improved by 32% after the treatment of ESOCA.
4. Discussion

In this work, the efficacy of an innovative optical clearing agent, ESCOA, is evaluated for improving the signal quality in IVFC measurement. Our results demonstrate that topical application of ESCOA on rat ear skin can greatly improve the signal quality in deep tissue and enhance the detection depth of IVFC in vivo. However, in Fig. 3(a), the signal intensity at a superficial depth of 80 μm is lower after the treatment by ESCOA than that by glycerol. Since the ESCOA can penetrate easier into skin than glycerol, which can reduce the scattering of ear skin and make the ear more transparent. The better the ear skin optical clearing efficacy is, the deeper the light penetrates. After the treatment of ESCOA, more fluorescence molecules in deeper tissue are excited, thus stronger signal is detected. It seems puzzling to understand the decrease in the fluorescence from superficial layer, which is currently under investigation.

Previously, 100% glycerol is usually applied on ear skin. On one side, the glycerol works as the couplant to decrease the propagation loss. On the other side, it has optical clearing efficacy. However, because of the induction of osmotic stress, OCAs may cause local hemostasis and even tissue necrosis after the application of high concentration of OCAs for a long period of time [28–31]. A previous study demonstrated that the application of 100% glycerol to the subdermis of hamster could block the blood flow in the venules [29]. Cheng et al. found out that the blood velocity was changed after the application of glycerol on rabbit dura matter [30]. Zhu et al. also found out that glycerol could reduce the local blood flow velocity or even block the vessels [31]. Therefore, the efficacy of glycerol was sub-optimal. Recently, we reported that topical application a mixture of PEG-400, fructose and thiazone (ESOCA) on the skin of mouse ear could enhance the transmittance without notable side effect on cutaneous blood vessels while no blood flow was blocked in the short term [24].

The full duration at half maximum (FDHM) represents the time that fluorescently labeled cells traverse the laser slit. The faster RBCs flow in the vessel, the shorter the FDHM is. With the same cumulative frequency, the FDHM in the case of ESCOA is less than that of glycerol (Fig. 6). Therefore the FDHM in the ESCOA-treated case mainly tends to distribute on the small-value portion by comparison, which means that the blood flow is faster. Therefore, as to the decrease in blood flow after application of glycerol, ESCOA might ameliorate this undesired impact in the long term.
Fig. 6. Full duration distribution at half maximum of IVFC signals after the treatment of OCAs for one hour. The FDHM indicates the speed of fluorescently labeled cells travelling through the laser slit. Thus a left shift to lower FDHM values indicates faster blood flow.

The reason why in vivo flow cytometer outperforms a confocal microscope for detecting circulating cells is improved signal to noise ratio (SNR). The sensitivity of the IVFC is expected to be significantly higher than that of a video-rate microscope for two reasons. First, because the IVFC detection scheme is non-imaging, all fluorescence photons from a cell will be detected by a single “pixel”. In comparison, in an imaging system (such as a microscope) the fluorescence from a cell is spread out over an area of roughly $10 \times 10 = 10^2$ pixels. Secondly, the bandwidth of the signal in the IVFC is in the kilohertz range (~1 msec signal pulse width), in contrast to a bandwidth of ~10 MHz for the video rate microscope. Therefore the SNR should improve by another factor of $(10^4)^{1/2} = 10^2$. Thus a theoretical overall improvement in sensitivity is $10^4$.

The study here focuses on the improvement of IVFC measurement by applying a novel optical clearing agent, ESOCA. It can improve the detection depth and signal quality in the deep skin of rat ear. It should be noted that topical treatment of glycerol does not increase the transmittance of rat ear noticeably for its limited penetration. After saline was used to wash the surface of skin, the transmittance spectrum almost returns to the initial state. It indicates that the penetration of glycerol into the dermis is very limited, which was also demonstrated previously [32]. The treatment of ESOCA makes the rat ear very transparent. Therefore, we can conclude that the significant improvement on IVFC measurement results from ESOCA. Optical clearing of ear skin induced by glycerol is limited, which is further eliminated by saline wash. With the development of novel optical clearing agents, IVFC may be useful for studying cancer metastasis not only in small animals, but also in large mammals and potentially human beings.

Acknowledgments

This work was supported by the grants of the National Major Scientific Research Program of China (Grants No. 2011CB910401, 2011CB910404 and 2012CB966800), the National Nature Science Foundation of China (Nos. 81171376, 91232710, 812111313 and 61227017), the Foundation for Innovative Research Groups of the NNSFC (Grant No. 61121004), the Research Fund for the Doctoral Program of Higher Education of China (No. 20110142110073), SJTU-UM Joint Grant (12X120010003), and Shanghai Science and Technology Committee (11DZ2211000). The authors are also grateful to Dr. Q. Chen and Dr. C. Xie for their help in animal care, R. Liu and Dr. Y. Suo for their suggestions.

#194323 - $15.00 USD  Received 22 Jul 2013; revised 4 Oct 2013; accepted 10 Oct 2013; published 17 Oct 2013 (C) 2013 OSA 1 November 2013 | Vol. 4, No. 11 | DOI:10.1364/BOE.4.002518 | BIOMEDICAL OPTICS EXPRESS 2526