J Fluoresc (2011) 21:1325–1329
DOI 10.1007/s10895-010-0828-5

SHORT COMMUNICATION

Quantitative Effect of Reducing Body Thickness on Visualizing Murine Deep Abdominal Lymph Nodes by In Vivo Fluorescence Reflectance Imaging

Yusuke Inoue · Makoto Watanabe · Shigeru Kiryu · Kuni Ohtomo

Received: 6 June 2010 / Accepted: 29 December 2010 / Published online: 12 January 2011

Abstract Scattering and absorption in the tissues are major problems for in vivo imaging based on a fluorescence reflectance imaging technique. We evaluated the quantitative relationship between body thickness and fluorescent signals from a deep abdominal source in intact mice. Mice were injected with quantum dots (peak emission, 800 nm) into the right rear footpad, and fluorescent signals from the iliac lymph node located deeply in the abdomen were assessed by fluorescence reflectance imaging. Stepwise compression of the mouse abdomen to reduce the body thickness was attained using a homemade simple device. The iliac node signals were weak and diffuse without compression but became stronger and more localized with decreasing body thickness. Using excitation light of approximately 710 nm wavelength, the lymph node/background contrast increased about 16 times with a 4 mm reduction in body thickness. Contrast enhancement was more evident using shorter wavelength excitation light. Overlying tissues profoundly affect signals from a deep source in fluorescence reflectance imaging. Our simple compression method may contribute to quantitatively assessing deep fluorescent sources.

Y. Inoue (✉)
Department of Diagnostic Radiology,
Kitasato University School of Medicine,
1-15-1 Kitasato, Minami-ku, Sagamihara,
Kanagawa 252-0374, Japan
e-mail: inoueys34@gmail.com

M. Watanabe · S. Kiryu
Department of Radiology, Institute of Medical Science,
University of Tokyo,
Tokyo, Japan

K. Ohtomo
Department of Radiology, Graduate School of Medicine,
University of Tokyo,
Tokyo, Japan

Keywords Fluorescence imaging · Mouse · Lymph node · Quantum dots · Attenuation

Introduction

In vivo fluorescence imaging (FLI) visualizes the amount and distribution of fluorescent substances in living animals and provides a variety of information depending on the fluorescent materials administered. Most FLI studies employ a fluorescence reflectance imaging technique in which excitation light is applied and emission light is detected from the same side of the animal [1]. Although fluorescence reflectance imaging offers whole-body images with a short acquisition time, this method has a weak point for visualizing fluorescent sources located deeply. Although light in the 600–900-nm wavelength range penetrates relatively long distances in biological tissues and is preferred for in vivo imaging [2], scattering and absorption is still a major problem in this range, causing signal attenuation and diffusion. The effects of absorption and scattering are much more severe for deep sources than for superficial sources because of the prolonged light path to and from the source. Assessing deep sources using fluorescence reflectance imaging usually requires surgical exposure of the target tissue, which complicates experimental procedures and disturbs repeated assessment in individual animals, which is a major advantage of in vivo imaging.

Semiconductor quantum dots (QDs) are bright, stable fluorophores with favorable properties, including variation in fluorescence emission and broad excitation spectra [3]. QDs are retained efficiently in the lymph node and suitable for sentinel lymph node mapping [4–9]. After subcutaneous injection into the rear footpad of mice, QDs are accumu-

lated mainly in the ipsilateral popliteal, sacral, iliac, and renal lymph nodes [10]. Although the iliac node is located deep within the abdomen, compression of the abdomen with tape enables the node to be visualized clearly [10]. Compression reduces the thickness of the tissues overlying the iliac node and, consequently, the effect of scattering and absorption during light transmission. Although compression with tape is convenient and effective for detecting deep sources, the degree of compression is difficult to control. The fluorescent signal intensity may vary depending on the difference in body thickness, which introduces errors in quantitative evaluation. In the present study, we constructed a simple device to compress the mouse abdomen to a predetermined thickness and, using the device, evaluated the quantitative relationship between abdominal thickness and fluorescent signals from the iliac lymph node. The aim of this study was to examine the feasibility of quantitative assessment using the compression technique and to quantitatively assess the effect of overlying tissues on in vivo FLI.

Materials and Methods

Animals

Female BALB/c mice (10 or 11 weeks old; SLC Japan, Hamamatsu, Japan) were used for the experiments. The mice were handled according to the guidelines of the Institute of Medical Science, University of Tokyo. The experiments were approved by the committee for animal research at the institution. Mice were given a purified diet (diet No. A10000; CLEA Japan, Tokyo, Japan) for at least 2 days before FLI to reduce autofluorescence from the intestine [10, 11]. Hair was removed prior to imaging with depilatory mousse (Epilat; Kracie, Tokyo, Japan).

CCD Camera

A cooled charge-coupled device (CCD) camera system (IVIS Imaging System 100; Xenogen/Caliper Life Sciences, Alameda, CA) equipped with a fluorescence kit (XFO-6; Xenogen/Caliper Life Sciences) was used. All images were acquired using a 775–825-nm passband emission filter (Chroma Technology, Rockingham, VT). Three different filters of 672.5–747.5 nm passband (Chroma Technology), 615–665 nm passband (Xenogen/Caliper Life Sciences), and 580–610 nm passband (Xenogen/Caliper Life Sciences) were used for excitation and are described here as long, middle, and short filters, respectively. Other imaging parameters were the field-of-view, 25 cm; binning, 2; and exposure time, 2 s.

Imaging Procedures

Six mice were subcutaneously injected with Qdot 800 ITK Carboxyl Quantum Dots (20 pmol in 10 μL; Invitrogen, Grand Island, NY), whose emission peak is 800 nm, in the dorsal side of the right rear footpad using a microsyringe, and a gentle massage was applied to the injection site for about 15 s.

In vivo FLI was performed 24 h after injection under isoflurane anesthesia. The homemade compression device was placed on the imaging table of the CCD camera system, and the mouse was placed on the device in the supine position (Fig. 1). The posterior limbs were fixed with colorless tape, and the right posterior limb was shielded with black, opaque tape to remove light from the injection site. First, a colorless, transparent plate was placed above the abdomen on 15-mm-high supports located on both lateral sides, and ventral images were acquired using the three different excitation filters successively. The plate neither touched the body surface nor compressed the mouse body. Next, 10-mm-high supports replaced the 15-mm-high supports, which resulted in mild compression and created a 10-mm-thick abdomen. Images were collected again for the compressed mouse using the three excitation filters. Then, FLI was repeated using supports with heights of 9, 8, 7, and 6 mm, leading to a stepwise reduction in body thickness. Thereafter, the mice were killed and the tissues corresponding to focal FLI signals indicative of accumulation in the right iliac lymph node were excised. The excised tissues and the remaining body were imaged with the CCD camera to confirm that the excised tissues were the sources of the focal signals.

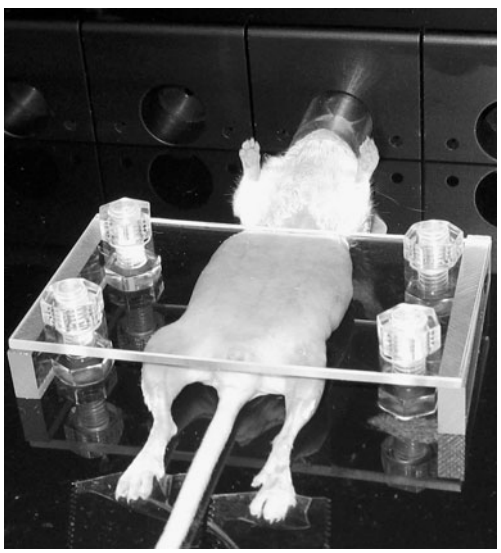

Fig. 1 A photograph of a mouse placed on the compression device. The right posterior limb was covered with black tape during the actual experiments

Data Analysis

The data were analyzed using Living Image software (version 3.2; Xenogen/Caliper Life Sciences). To evaluate the relationship between body thickness and signal intensity from the iliac lymph node, a fixed-size circular region of interest (ROI) was set over the focal signal corresponding to the iliac lymph node, and the maximum signal in the ROI was determined (LN_{max}, p/s/cm^2/sr). A fixed-size rectangular ROI was placed for the left flank, lateral to the lymph node ROI and near the body contour, as a background region, and the average signal in the ROI was computed (BKG_{ave}). The ROIs were set on all images acquired at various body thicknesses using the three excitation filters. The ROI positions were identical among the three images acquired successively with the different excitation filters and adjusted for images acquired at different body thicknesses. LN_{max}/BKG_{ave} was calculated to define the lymph node/background contrast, and the contrast was normalized using that obtained at a 10 mm body thickness for each mouse and for each excitation filter. Additionally, the lymph node margin was defined using 50% of the maximum signal as a threshold, and the area of the lymph node signal (mm^2) was determined. Because the automatic ROI setting requires sufficient lymph node/background contrast, the area was estimated only on the images acquired with compression and the long excitation filter.

Results

Data acuquisition took about 15 min per mouse. In vivo FLI using the long excitation filter demonstrated focal signals in the abdomen in all six mice, and FLI of the excised tissues revealed that the signals originated from the right iliac lymph node. The signals were weak and diffuse without compression, and became stronger and more localized with decreasing body thickness (Fig. 2). The mean lymph node/background contrast was 5.8 without compression and 254.2 at a 6 mm body thickness (Fig. 3a). The mean contrast normalized for that at a 10 mm thickness was 0.36 without compression and 15.6 at a 6 mm thickness (Fig. 3b). The mean area of the lymph node signal was 15.4 mm^2 at a 10 mm thickness and 3.8 mm^2 at a 6 mm thickness (Fig. 4).

The normalized lymph node/background contrast at a small body thickness was larger using shorter wavelength excitation light, indicating a larger dependence of contrast on body thickness (Fig. 3b). The contrast without normalization was lower using the short excitation filter than using the long or middle filters, regardless of body thickness. At a

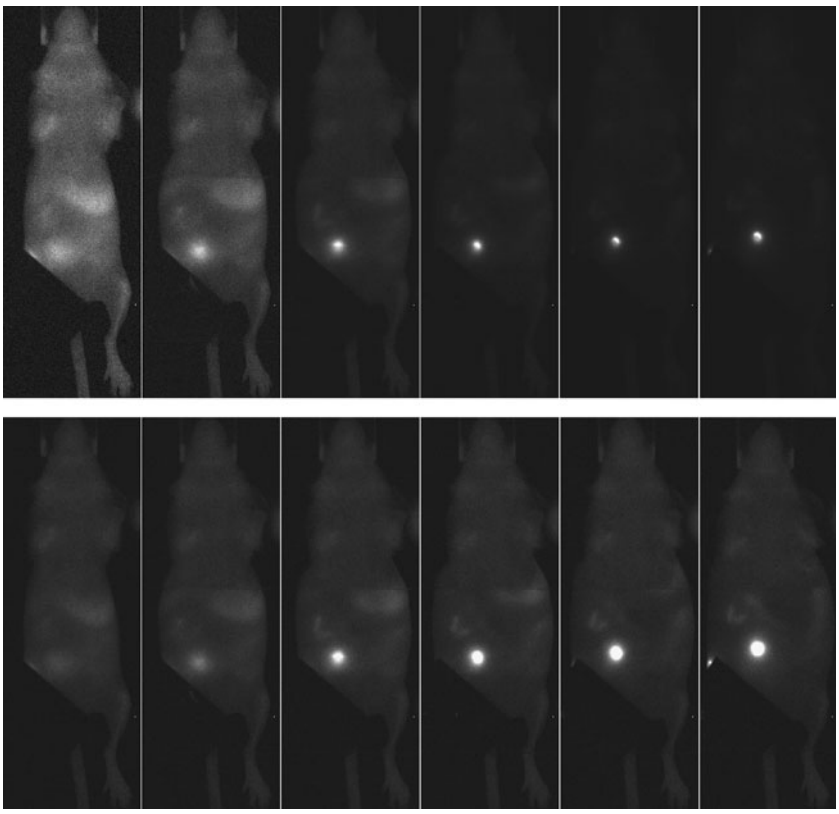

Fig. 2 Representative images of in vivo FLI. Ventral images acquired without compression and with body thicknesses of 10, 9, 8, 7, 6 mm are presented from left to right in each row. In the upper row, the gray scale was adjusted for each image to display the iliac lymph node. In the lower row, the same gray scale was applied to all images. The right inguinal lymph node is noted near the black tape on some images

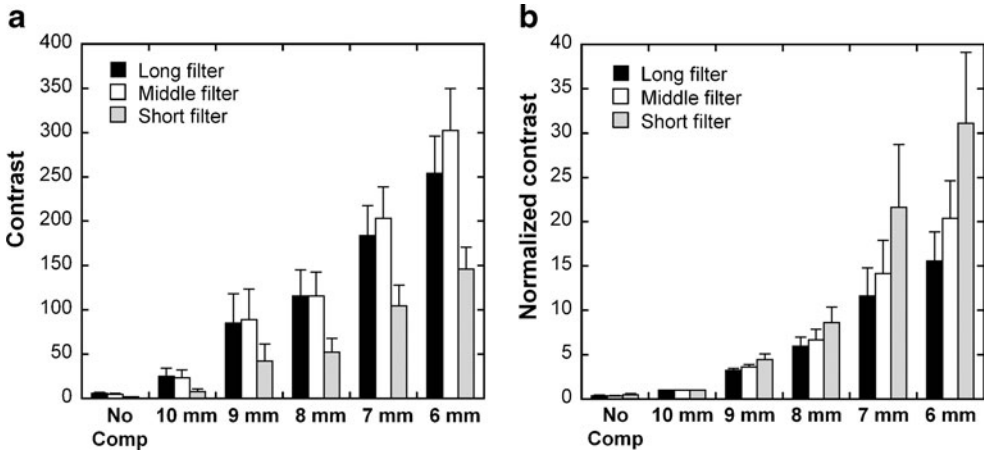

Fig. 3 The effect of compression on the lymph node/background contrast before (**a**) and after (**b**) normalization using the value obtained at a 10 mm body thickness. On the x-axis, "No Comp" indicates no compression and "10 mm" indicates a body thickness of 10 mm. Error bars indicate standard errors

small thickness, the contrast was slightly higher using the middle excitation filter than using the long excitation filter.

Discussion

Compressing the mouse abdomen decreases the effect of scattering and absorption in the overlying tissues and improves visualization of the iliac lymph node by in vivo FLI [10]. In this study, we evaluated the quantitative relationship between body thickness and fluorescent signals from the iliac node using a simple device. The device compresses the mouse abdomen with a plate set at preindicated heights. Stepwise, controlled compression was attained using this simple device, which allowed for a quantitative evaluation of depth-dependent changes in fluorescent signals. No signs of poor health was noted after the compression imaging. Repeated compression did not result in systematic fluorescence intensity changes (data not shown), and the effect of compression on QD retention in the lymph node, such as accelerated washout, was not suggested. Accumulation in the lymph node is similar between 3 and 24 h postinjection [10]. In this study, the waiting time was set at 24 h to reduce changes in accumulation during the 15 min of data acquisition.

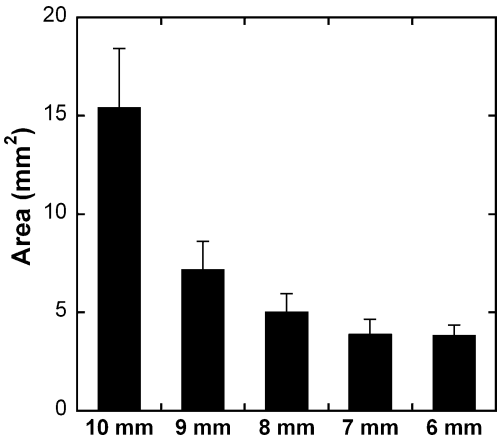

Fig. 4 The effect of compression on the lymph node signal area. On the x-axis, 10 mm indicates a body thickness of 10 mm. Error bars indicate standard errors

The lymph node/background contrast increased with decreasing body thickness. Using a 672.5–747.5 nm passband excitation filter, the contrast increased about 16 times with a 4 mm reduction (from 10 to 6 mm) in body thickness, confirming profound light attenuation in the tissues. Although enhanced fluorescent signals improve sensitivity to deep sources, small differences in the degree of compression may cause large errors in quantitative estimates. Compressing to a preindicated thickness appears to contribute to decreasing variability in the experimental results.

Because light scattering produces fringe signals, resulting in an unclear margin for the deep source, subjective measurement of the size of a fluorescent region is unreliable. We defined the lymph node signal area using a thresholding method and demonstrated a reduction in the area with decreasing body thickness. A decrease in light diffusion due to a decrease in overlying tissues, in addition to enhanced signal intensity, leads to distinct visualization of deep fluorescent structures. Reducing the fluorescent area by compression appears to facilitate discrimination between two fluorescent sources located closely and may aid in distinguishing between normal and enlarged lymph nodes.

The QDs show very broad excitation spectra [3]. Although the extinction coefficient is larger at shorter wavelengths, longer wavelength light may excite QDs more effectively in in vivo FLI because of the severe attenuation of short wavelength light in biological tissues [12]. In the present study, contrast enhancement by compression was more prominent for shorter excitation light, which appears to be attributable to more severe attenuation in the tissues. Although the lymph node/background contrast was similar at a large body thickness between the long and middle excitation filters, it was higher using the middle filter at a small

body thickness. Reducing attenuation in the tissues appears to increase the relative contribution of the extinction coefficient for determining the fluorescent signals. Our results highlight that signals obtained from deep sources after QD injection depend on light transmission through the tissues and the extinction coefficient, and that the optimal wavelength for excitation may vary according to the overlying tissues.

Conclusions

We evaluated the quantitative relationship between body thickness and fluorescent signals from deep abdominal lymph nodes using a simple compression device and demonstrated an increase in fluorescence intensity and a decrease in fluorescence area with decreasing body thickness. Enhanced fluorescence intensity was more prominent for shorter wavelength excitation light. The compression method described here is expected to contribute to the quantitative assessment of deep fluorescent sources.

Acknowledgments We thank Shin-ichi Kaneda, RT, Department of Radiology, Institute of Medical Science, University of Tokyo, for his help in making the compression device.

Conflict of interest No conflict of interest exists in connection with this article.

References

1. Ntziachristos V, Bremer C, Weissleder R (2003) Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging. Eur Radiol 13(1):195–208
2. Weissleder R (2001) A clearer vision for in vivo imaging. Nat Biotechnol 19(4):316–317
3. Hotz CZ (2005) Applications of quantum dots in biology: an overview. Meth Mol Biol 303:1–17
4. Robe A, Pic E, Lassalle HP, Bezdetnaya L, Guillemin F, Marchal F (2008) Quantum dots in axillary lymph node mapping: biodistribution study in healthy mice. BMC Cancer 8:111
5. Frangioni JV, Kim SW, Ohnishi S, Kim S, Bawendi MG (2007) Sentinel lymph node mapping with type-II quantum dots. Meth Mol Biol 374:147–159
6. Ballou B, Ernst LA, Andreko S, Harper T, Fitzpatrick JA, Waggoner AS, Bruchez MP (2007) Sentinel lymph node imaging using quantum dots in mouse tumor models. Bioconjug Chem 18 (2):389–396
7. Kim S, Lim YT, Soltesz EG, De Grand AM, Lee J, Nakayama A, Parker JA, Mihaljevic T, Laurence RG, Dor DM, Cohn LH, Bawendi MG, Frangioni JV (2004) Near-infrared fluorescent type II quantum dots for sentinel lymph node mapping. Nat Biotechnol 22(1):93–97
8. Hama Y, Koyama Y, Urano Y, Choyke PL, Kobayashi H (2007) Simultaneous two-color spectral fluorescence lymphangiography with near infrared quantum dots to map two lymphatic flows from the breast and the upper extremity. Breast Cancer Res Treat 103:23–28
9. Kobayashi H, Hama Y, Koyama Y, Barrett T, Regino CA, Urano Y, Choyke PL (2007) Simultaneous multicolor imaging of five different lymphatic basins using quantum dots. Nano Lett 7 (6):1711–1716
10. Inoue Y, Kiryu S, Watanabe M, Oyaizu N, Ohtomo K (2010) Fluorescence lymph node mapping in living mice using quantum dots and a compression technique. J Fluoresc 20 (2):599–606
11. Inoue Y, Izawa K, Kiryu S, Tojo A, Ohtomo K (2008) Diet and abdominal autofluorescence detected by in vivo fluorescence imaging of living mice. Mol Imaging 7(1):21–27
12. Lim YT, Kim S, Nakayama A, Stott NE, Bawendi MG, Frangioni JV (2003) Selection of quantum dot wavelengths for biomedical assays and imaging. Mol Imaging 2(1):50–64