Use of lipophilic near-infrared dye in whole-body optical imaging of hematopoietic cell homing

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Abstract. We develop an optical whole-body imaging technique for monitoring normal and leukemic hematopoietic cell homing *in vivo*. A recently developed near-infrared (NIR) lipophilic carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) is used to safely and directly label the membranes of human leukemic Pre-B ALL G2 cell lines as well as primary murine lymphocytes and erythrocytes. DiR has absorption and fluorescence maxima at 750 and 782 nm, respectively, which corresponds to low light absorption and autofluorescence in living tissues. This allows us to obtain a significant signal with very low background level. A charge-coupled device (CCD)-based imager is used for noninvasive whole-body imaging of DiR-labeled cell homing in intact animals. This powerful technique can potentially visualize any cell type without use of specific antibodies conjugated with NIR fluorescent tag or loading cells with transporter-delivered NIR fluorophores. Thus, *in vivo* imaging based on NIR lipophilic carbocyanine dyes in combination with advanced optical techniques may serve as a powerful alternative or complementation to other small animal imaging methods. © *2006 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2364903]

Noninvasive, *in vivo* imaging of targeted cells is a promising field for studying cell behavior and movement in animal models. Intravital microscopy and whole-body fluorescent optical imaging are among the most widely used approaches for visualizing naive or genetically modified cells *in vivo*.[1–3] However, advances of fluorescent microscopy are not necessarily applicable for whole-body imaging. For example, fluorescent pigments and reporter genes that have been predominantly used for *in vivo* imaging emit light in the visible range of spectrum. This is associated with significant limitations, such as tissue light absorption, scattering, and autofluorescence.[4] In contrast, dyes fluorescing in the near-infrared (NIR) band should be free of this handicap.[5] To the best of our knowledge, up until now no study has been reported regarding the application of NIR lipophilic dyes for whole-body optical imaging of hematopoietic cell homing.

To study homing patterns of hematopoietic cells, different cell types were utilized: human leukemic Pre-B ALL G2 cells (G2L), primary mouse lymphocytes (ML), and fresh murine red blood cells (RBC). A recently introduced carbocyanine lipophilic NIR fluorescent membrane dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR, Invitrogen) was used for labeling the cells.[6] This fluorophore is excited at 750 nm and has an emission peak at 782 nm. Cells were incubated with DiR [1×10^7 cells in 10-ml phosphate buffered saline (PBS) containing 3.5-μg/ml dye and 0.5% ethanol] for 30 min at 37°C. Thereafter, cells were washed twice with PBS and the viability of labeled G2L and ML cells was verified by trypan blue staining. Labeled cells were then injected intravenously into NOD/SCID mice, as well as into CD1 nude mice, which were previously anesthetized by intraperitoneal injection of 35 μl of a mixture of ketamine (Rhone Merieux, Lyon, France) and xylazine 2% (Vitamed, Hedera, Israel) (85:15 vol:vol). Xenogen's IVIS® 100 Series Imaging System (Alameda, California) and Olympus SZX12 (Tokyo, Japan) microscope, coupled with a Pixelfly QE (PCO, Kelheim, Germany) charge-coupled device (CCD) camera, were used to monitor DiR labeled G2L and ML localization within live, as well as sacrificed, animals. Imaging was performed at 16-, 24-, and 48-h post cell injection. The excitation and emission filter set in the IVIS was 710 to 760 nm and 810 to 860 nm, respectively. The emission filter with the wavelength closest to the emission peak of the dye was selected among available filters in the standard configuration of IVIS. An Olympus SZX12 microscope was equipped with a home-built illumination system with a 720- to 750-nm excitation filter and a 780- to 850-nm emission filter, which was placed between the lens and camera. Image processing and data analysis were performed using Living Image 2.5 software and ImageJ version 1.34 (Wayne Rasband, National Institutes of Health, Maryland). All experimental results were repeated on at least three different animals and representative pictures are shown.

To visualize *in vivo* cell homing, DiR labeled G2L cells or ML were injected intravenously into mice. As depicted in Fig. 1(a), no signal was detected in intact nude mice that had been injected with unlabeled cells. In control experiments, injection of free dye in several concentrations resulted in nonspecific distribution of the fluorescent signal all around the body with no sign of any specific pattern. In animals injected with labeled cells, a general increase of NIR fluorescent signal from the whole body started to develop immediately after injection. During the first 6 h after injection, a moderate increase in signal intensity was observed in the area of spleen and liver, and homing to specific organs started (data not shown). The fluorescence pattern was clearly detectable in 16 h, reached maximal intensity in 24 h, and persisted up to 48 h. Figures 1 and 2 demonstrate the fluorescent signal at its maximum—24 h after injection. After 48 h, the signal began to decay,
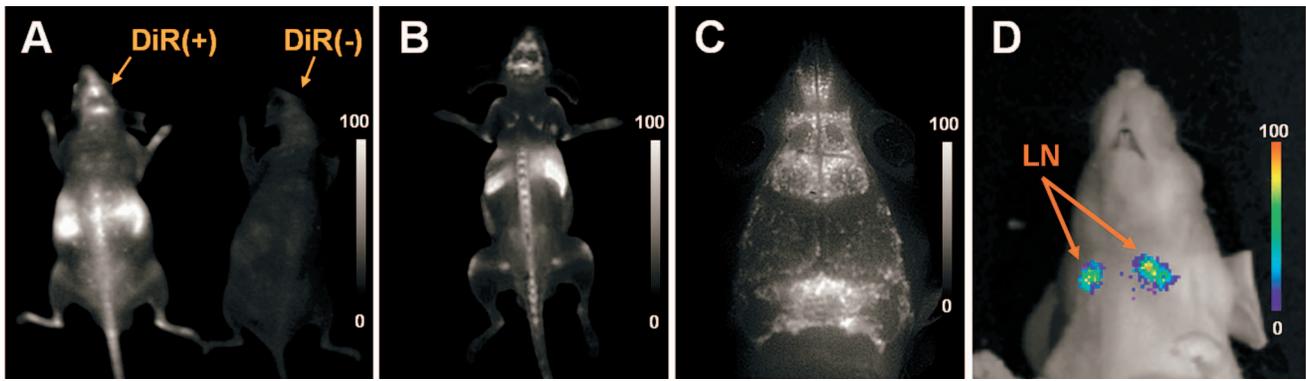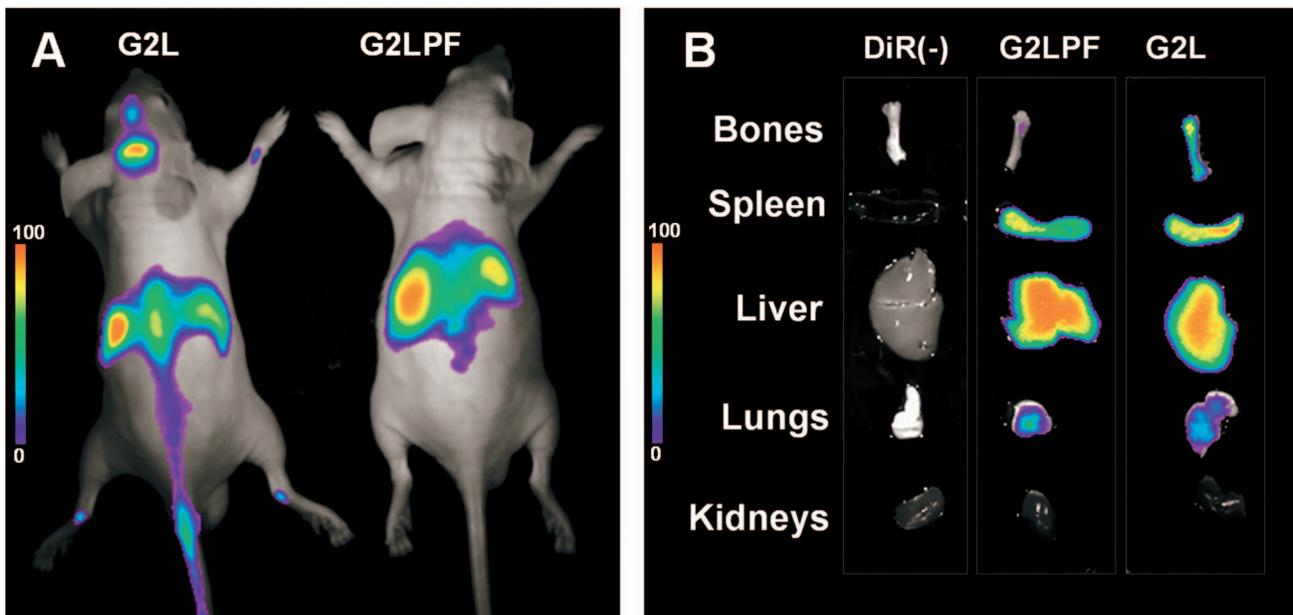
Fig. 1 Near-infrared fluorescent (NIRF) images of DiR labeled cells homing in mice. (a) Noninvasive monochrome NIRF images of unlabeled and DiR labeled G2L cell homing in CD1 nude mice, 24 h after transplantation of 10^7 cells. DiR(+) cells were labeled with DiR. DiR(−) cells were not labeled with DiR. (b) Monochrome NIRF image of a sacrificed CD1 nude mouse (skin was removed) 24 h after transplantation of 10^7 DiR labeled G2L cells. (c) Monochrome NIRF image of the cranium of sacrificed CD1 nude mouse (skin was removed) 24 h after transplantation of 10^7 DiR labeled G2L cells. (d) Noninvasive color-coded contrast enhanced NIRF image of DiR labeled lymphocyte homing overlaid on a photographic image. LN -superficial cervical lymph nodes of CD1 nude mice 24 h after transplantation of 10^7 cells. The cutoff of low intensity light was adjusted to make a detected autofluorescent signal below the threshold. Monochrome gradual bar and color gradual bar show intensity of fluorescence in arbitrary units.

remaining detectable although markedly reduced even at 72 to 96 h (data not shown). In particular, abundant fluorescence was detected in the bone marrow within the spinal column, limbs, and skull. In addition, a strong fluorescent signal was detected in the spleen, liver, and lymph nodes [Figs. 1(a) and 1(d)], reflecting cell accumulation in these specific lymphatic organs. Examination of sacrificed animals after skin removal or isolated organs revealed a stronger signal compared to living mice, and additional regions of previously undistinguishable fluorescence were identified. For example, a weak signal was detected in the scapulae and pelvic bones

[Fig. 1(b)]. In addition, regions in the skull bones infiltrated with labeled cells could be easily distinguished from noninfiltrated regions [Fig. 1(c)]. These findings support previous reports describing lymphocyte homing into the bone marrow and lymphatic organs using other techniques.[7,8] Thus, it may be concluded that DiR labeling does not impair *in vivo* homing of human G2L cells and ML.

G2L or ML homing, especially into the bone marrow, is an active process typical only of living cells. Therefore, to distinguish between active homing and passive cell transfer, cells were fixed with 3% paraformaldehyde in phosphate buffered

Fig. 2 Effect of paraformaldehyde (PF) on the efficiency of G2L cell homing. (a) Noninvasive color-coded near-infrared fluorescent (NIRF) images of cell homing in CD1 nude mice overlaid on a photographic image 24 h after transplantation of 10^7 DiR labeled G2L cells (G2L) or fixed DiR labeled G2L cells (G2LPF). (b) Color-coded NIRF image of the internal organs of CD1 nude mice overlaid on a photographic image 24 h after transplantation of 10^7 DiR labeled G2L cells. DiR(−) indicate animals injected with G2L cells unlabeled with DiR. The cutoff of low intensity light was adjusted to make a detected autofluorescent signal below the threshold. Color bars show intensity of fluorescence in arbitrary units.

saline (PBS) for 15 min prior to labeling and injection into mice. Unfixed cells were used as a control. In the experiments in which fixed cells were used, fluorescence was found exclusively in the spleen and liver, suggesting that either dead cells or released dye accumulated in these organs [Fig. 2(a)]. Moreover, a markedly reduced signal, compared to nonfixed DiR-labeled cells, was observed in the bones [Fig. 2(b)]. Notably, no fluorescent signal was detected in the kidneys. This observation suggests that kidneys could serve as a reference organ to which neither G2L nor ML cells home.

Thus, our results for the first time demonstrate that NIR lipophilic carbocyanine dyes can be a powerful tool for both normal and leukemic cell labeling for whole-body optical imaging, providing an efficient alternative to genetic manipulations, staining with other fluorescent reagents, and radioactive cell labeling. Transfection of immune or hematopoietic cells with fluorescent or luminescent reporter genes (for example, luciferase, green fluorescent protein, or red fluorescent protein) prior to their injection is a technically complicated procedure giving relatively low yield that is difficult to standardize.[9,10] Recently, much attention has been drawn to organic dyes and quantum dots (QDs), NIR light, allowing photons to penetrate up to several centimeters into living tissue.[11–13] Despite optical advantages of QDs compared to organic dyes, there are still several limitations and concerns regarding the use of QD-bioconjugates for *in vivo* cellular imaging.[14,15] A major problem is the difficulty of achieving a high efficiency of labeling during short *in-vitro* procedures. This is especially critical when hematopoietic cells are utilized, as they are very sensitive to prolonged *ex-vivo* treatment. In contrast, the proposed method of cellular staining is rapid (15 to 40 min incubation) and therefore comparatively harmless to cells. Effective labeling of membrane phospholipids by DiR or its analogs in combination with spatial and temporal resolutions (due to optical modality of detection), makes this technique a particularly attractive candidate for *in vivo* tracking hematopoietic cell movement and homing. This technique can be a cost-effective alternative or complementary approach to other imaging modalities in small animal experimentation.

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