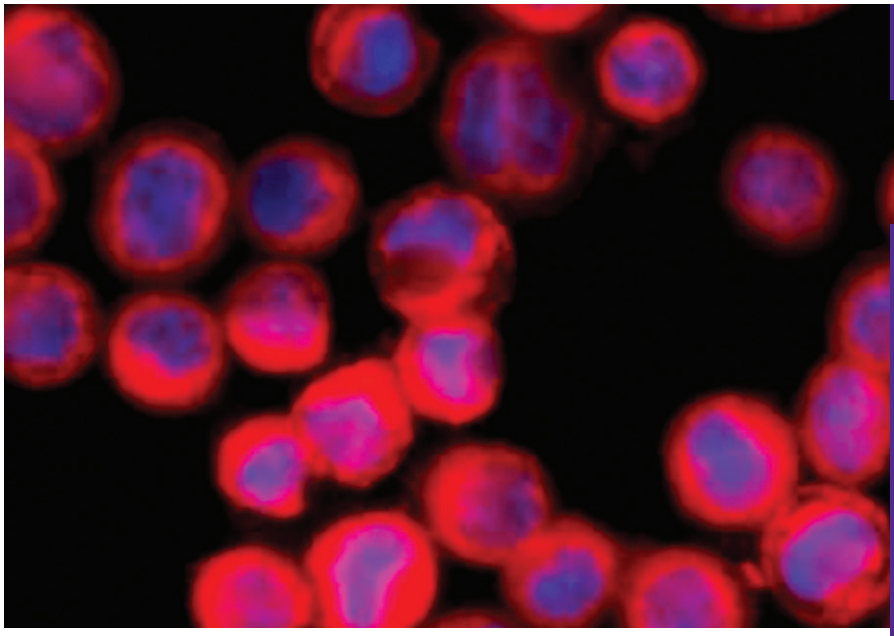

APPLICATION NOTE

VivoTrack 680

Author

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A method of NIR fluorescent cell-labeling for *in vivo* cell tracking

Abstract

Fluorescent dyes have been used for many years to label cells for microscopy studies, and a variety of dyes in the visible fluorescence spectrum are available to label different cellular compartments and organelles. Efficient delivery of the fluorophore to the cell without excessively modifying surface proteins or perturbing cell function is the major biotechnological challenge. In addition, researchers have taken on the challenge of *in vivo* imaging, focusing on near infrared (NIR) dyes that fluoresce in a spectral region better suited for *in vivo* imaging due to reduced background and higher tissue penetration. Here we introduce VivoTrack™ 680, a novel NIR dye that has a long aliphatic tail to facilitate intercalation into the lipid bilayer of the cell. This agent can label a variety of cells brightly and uniformly without requiring harsh organics such as DMSO or ethanol, chemicals known to affect cell function. The labeled cells retain excellent viability and normal proliferative capacity, and significant signal is retained in dividing cells *in vitro* for more than 7 days. Many different cell types can be labeled for *in vivo* transfer or implantation, and *in vivo* utility is illustrated by non-invasively tracking fluorescent primary macrophages to sites of inflammation by NIR fluorescent imaging.

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MATERIALS AND METHODS:

Fluorescent Cell Labeling Dye

VivoTrack 680 is a near infrared (NIR) dye with an long aliphatic tail that facilitates integration into the cell membrane for the purpose of identifying cells *in vitro* or tracking them *in vivo*. This agent provides a simple and rapid method for labeling cells without affecting cell viability, proliferation, and function.

Table 1.
Basic Properties of VivoTrack 680 Fluorescent Cell Labeling Agent

	VivoTrack 680
Agent Type	Lipophilic dye
Molecular Weight	1173 g mol^{-1}
Absorbance	676 nm
Fluorescence emission	696 nm

Cells and Cell Lines

The mouse leukemic monocyte macrophage cell line RAW 264.7, was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in 75 cm^2 flasks. Exponentially growing cells between passages 1-10 were used for all experiments. Cells were labeled with VivoTrack 680, as described below and analyzed by flow cytometry (Becton Dickinson LSRII) and fluorescence microscopy (Zeiss Axioskop 2 MOT Plus, Oberkochen, Germany) using appropriate lasers and filters for detection of 680 nm wavelength. Cell nuclei were stained with DAPI (blue).

Mouse thioglycollate elicited peritoneal macrophages were also used as a source of primary cells for labeling and transfer studies. Briefly, a thioglycollate solution was prepared by boiling 3% Brewer thioglycollate medium in distilled water followed by storage at room temperature. Normal BALB/c mice were injected intraperitoneally with 1.5 mL of thioglycollate medium per 25 g body weight. This injection induced a proinflammatory response, generally yielding ~1 x 10^7 macrophages per mouse 3 days later. These cells were harvested from sacrificed animals by peritoneal lavage using 3 separate washes of the peritoneum for a total of 10 mL PBS. Subsequently, cells were washed with 1 X PBS and counted for labeling. Red blood cells were lysed as necessary.

VivoTrack Labeling of Cells

1. Wash the cells of interest once with sterile PBS or serum-free medium to remove serum proteins and lipids that may interfere with cell labeling.
2. Discard the supernatant and resuspend the cells (up to 250 x 10^6 cells/mL) in 2.0 mL of PBS in a 50 mL sterile conical tube.
3. Dissolve VivoTrack 680 (1 g) in 1.2 mL of warm sterile PBS (37 °C) and mix by vortex until completely dissolved. This will yield 2.0 mL of the labeling agent.
4. Add 2.0 mL of the cell labeling solution to 2.0 mL of cells, and mix immediately by gentle vortexing. *[Note: It may be required to optimize dose depending on the cells to be labeled.]*
5. Incubate the cells for 15 min at room temperature, protected from light.
6. Dilute the cells for washing by adding 15-20 mL sterile RT PBS containing 1% FBS or complete medium, depending on your ultimate use for the cells.
7. Wash the cells 3 times with RT PBS containing 1% FBS to remove excess cell labeling agent. A final resuspension with sterile PBS alone can be used to decrease the FBS levels in the cell preparation.
8. Count, culture, or transfer cells as required by the application.

VivoTrack 680 is highly soluble in aqueous solution and, thus, does not need to transition from organic to aqueous solution upon addition to the cell suspension. This allows quick dispersion into the cell medium, and very uniform cell labeling is generally achieved.

In Vivo Macrophage Tracking

Mouse carrageenan-induced paw edema, a model of acute neutrophil-mediated inflammation (Figure 1), was used for the purpose of recruiting macrophages to inflamed paw tissue. Briefly, BALB/c mice received an intravenous injection of 5 x 10^6 VivoTrack 680-labeled thioglycollate-elicited peritoneal macrophages to provide a reservoir of fluorescent-labeled cells available for recruitment in response to the carrageenan stimulus. Twenty-four hours later, each mouse received a 30 mL injection of PBS (left paw) and 1% carrageenan (right paw). Some mice were depilated completely to allow fluorescent imaging of whole body biodistribution of labeled macrophages at 24 h. Optimal macrophage recruitment occurred within 3 h of carrageenan injection (i.e. 27 h following macrophage injection) and this was easily detected and quantified in paws of un-depilated mice.

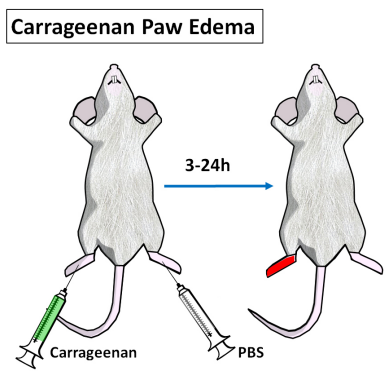

Figure 1.

Imaging Data Analysis

Epifluorescence (2D) images were captured at 3 h post-carrageenan injection by the IVIS® Spectrum CT imaging system (PerkinElmer, Waltham, MA). The FMT 4000 (Fluorescence Molecular Tomography) imaging system (PerkinElmer, Waltham, MA) was used to capture both epifluorescence and tomography [3D] datasets. To enable transillumination of the paws for tomographic imaging, paws were placed over an index-matching block.

Imaging datasets for the IVIS Spectrum were visualized and quantified by Living Image® 4.3.1 software, and FMT data was analyzed by FMT system software (TrueQuant™ v4.0), both from PerkinElmer (Waltham, MA). Regions of interest (ROI), 2D and 3D as necessary, were drawn encompassing each paw, with the PBS injected paws serving as the negative controls.

Introduction and Results

Understanding the pathophysiology of disease processes at the cellular and biochemical level is a major challenge in basic research and drug development. The complexity of these processes, and the difficulty of recapitulating this complexity *in vitro*, raises the importance of robust and quantitative *in vivo* techniques to study specific cellular players in disease progression. In inflammation, for example, the ability to observe and quantify the recruitment of leukocytes from the blood to sites of tissue infection or trauma is crucial for understanding the induction of acute or chronic inflammatory tissue destruction. Fluorescent dyes have been invaluable tools for this type of cell monitoring *in vitro* and *in vivo*, but today there remains the need for fluorescent labeling agents that can provide 1) uniform cell labeling, 2) long cellular residence time, 3) minimal impact on cell viability and function, and 4) a near infrared wavelength for optimal light penetration of tissue.

The VivoTrack 680 cell labeling agent provides a simple and easy NIR membrane labeling tool that is specially formulated for excellent solubility in the absence of organic solvents like ethanol or DMSO. This agent intercalates into cell membranes (primary macrophages and RAW264.7 cells here), providing a bright label by which cells can be tracked *in vivo*. A panel of studies determined that cells were consistently and brightly labeled as assessed by both flow cytometry and fluorescent microscopy, and cells retained excellent viability and proliferation. Primary mouse macrophages were readily tracked to a site of acute inflammation.

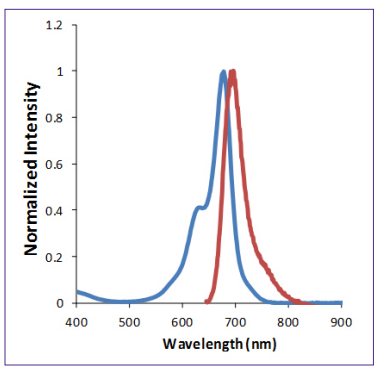

Figure 2.
VivoTrack 680 absorption and emission spectra show peak absorption at 676 nm and peak emission at 696 nm.

VivoTrack 680 In Vitro Characterization

Table 1 provides the physical-chemical characteristics and the absorbance and emission maxima, and Figure 2 shows the full spectral profile of VivoTrack 680. Cells are labeled brightly, with good cell-to-cell consistency, as shown by labeling of both a monocyte/ macrophage cell line (RAW 264.7), as well as primary thioglycollate-induced peritoneal macrophages (Figure 3).

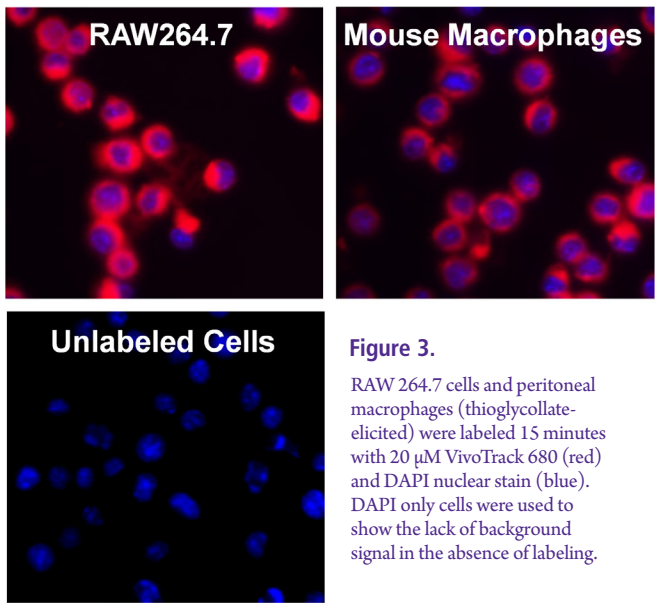

Figure 3.
RAW 264.7 cells and peritoneal macrophages (thioglycollate-elicited) were labeled 15 minutes with 20 µM VivoTrack 680 (red) and DAPI nuclear stain (blue). DAPI only cells were used to show the lack of background signal in the absence of labeling.

The brightness of cell labeling by VivoTrack 680 was determined by flow cytometry using the LSR II system (Becton Dickinson, Franklin Lakes, NJ) modified to include a 660 nm laser for NIR imaging. Cell proliferation/viability was assessed by two independent methods; WST assay and BrdU incorporation kits (Roche Diagnostics GmbH, Mannheim, Germany). Figure 4 shows that labeling of RAW264.7 cells with VivoTrack 680 yields bright cells with no effect on viability as assessed by both WST and BrdU incorporation assays.

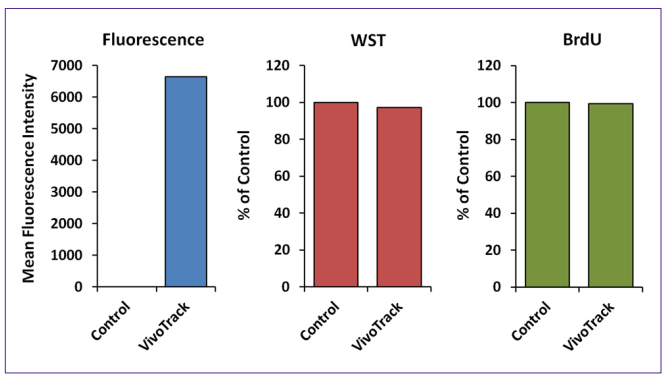

Figure 4.
RAW 264.7 cells were assessed for brightness and viability after 15 minute incubations with either vehicle or VivoTrack 680. Flow cytometry was used to assess fluorescence, and microplate-based assays were used to quantify viability.

Rapidly dividing cells are expected to lose membrane fluorescent signal over time, however signal is readily detectable for at least 7 days when labeling RAW264.7 cells with VivoTrack 680 (Figure 5). Fluorescence retention kinetics are related to the rate of cell division, and, thus, may depend on the type of cell labeled.

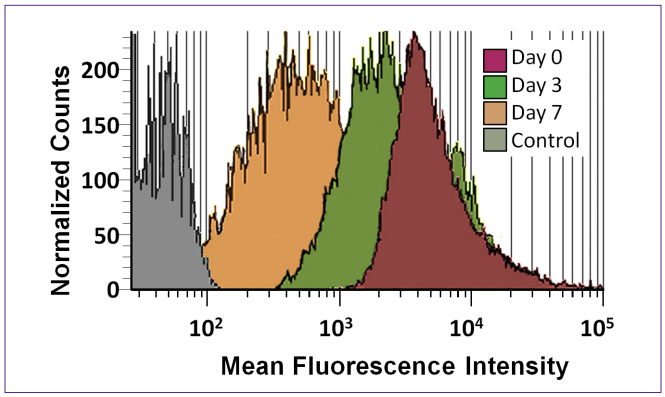

Figure 5.

The effect of RAW 264.7 cell proliferation on fluorescence levels after VivoTrack 680 labeling was assessed by maintaining cells in culture for up to seven days. Cells were assayed on days 0, 3, and 7, revealing a consistent rate of loss proportional to the overall increase in cell numbers.

VivoTrack 680 In Vivo Characterization

To assess the *in vivo* performance of VivoTrack 680 labeled macrophages, we used a simple mouse model of acute neutrophil-driven inflammation, carrageenan-induced paw edema. Briefly, thioglycollate-elicited macrophages from donor BALB/c mice were labeled with VivoTrack 680 and then transferred intravenously (5 x 106 cells/mouse) into normal BALB/c mice. The labeled cells were allowed to accumulate in the liver and lungs for 24 h, and then the recipient mice were injected with carrageenan (right footpad) and PBS (left footpad). The active site of inflammation was used as a means to recruit inflammatory cells, including the VivoTrack 680 labeled macrophages injected 24 h earlier.

We used non-invasive fluorescence imaging and IVIS Spectrum imaging systems to assess these changes three hours after carrageenan injection (Figure 6). Macrophages selectively trafficked into inflamed paws and accumulated there, showing statistically significant elevations in fluorescence. As expected, the peak macrophage influx (3 – 24 h) accounted for ~0.4-2% of the total injected cells (20-100,000 cells/mouse).

The kinetics of labeled macrophage accumulation in inflamed paws

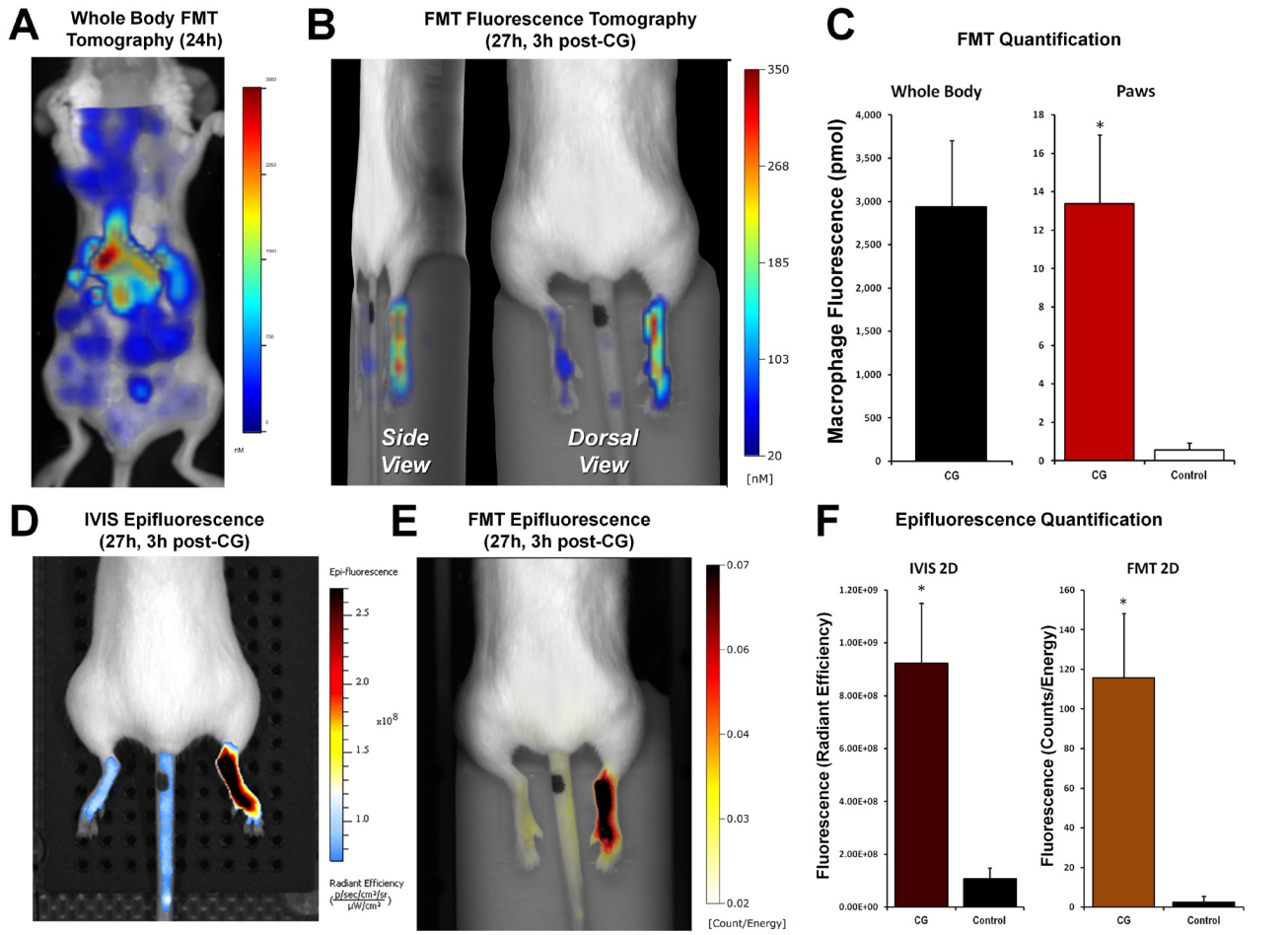

Figure 6. BALB/c mice received an intravenous injection of labeled thioglycollate-elicited peritoneal macrophages to provide a reservoir of fluorescent-labeled cells available for recruitment in response to an inflammatory stimulus. One cohort of mice was depilated completely to allow FMT 4000 tomographic imaging of whole body biodistribution of labeled macrophages at the time of carrageenan injection (A) for comparison to tomographic paw imaging 3h after carrageenan (CG) injection (B). FMT quantification shows that the paw signal is approximately 0.4% of the total body signal, indicating that ~20,000 cells were recruited to the inflamed paw (C). Epifluorescence assessed by IVIS (D) and FMT (E) each offer a rapid screening approach for the measurement of fluorescent macrophage recruitment to the inflamed paws (F), but not to the control, PBS-injected paws. Quantification of fluorescence datasets was performed with some modest thresholding to remove non-specific background fluorescence.

was assessed by imaging mice at multiple times after CG injection (Figure 7). Changes in paw thickness over time were also assessed by engineer's micrometer, indicating a peak in inflammation at 3 h which decreased steadily by 72 h (Figure 7A). A lower level chronic phase of paw swelling was observed between 72 and 168 h, perhaps reflecting a transition from acute neutrophilia to chronic host macrophage accumulation. The kinetics of the recruitment of injected fluorescent macrophages, measured by epifluorescence imaging, also peaked at 3 h and declined steadily until 168 h (Figure 7B), aligning well with peak inflammation and the likely secretion of cytokines and chemokines. Signal loss was attributed to macrophage efflux and/or loss of fluorescence due to in situ proliferation or death. This model would offer an ideal approach to assessing drugs or antibodies that affect chemokines or adhesion molecules involved in macrophage recruitment.

Summary

PerkinElmer has developed a near infrared fluorescent cell labeling agent, VivoTrack 680, that can generate brightly-labeled and highly viable cells suitable for detection and longitudinal tracking *in vivo*. As cell migration is critical for many important *in vivo* biological functions, this labeling approach has the potential to provide a useful tool for research in inflammation, immunology, and stem cells.

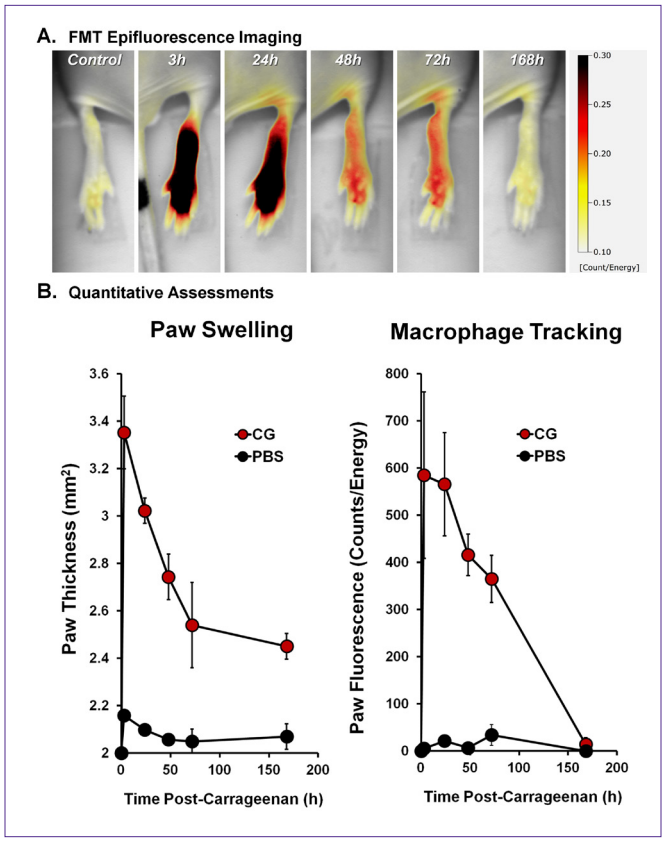

Figure 7.

A representative BALB/c mouse recipient of VivoTrack 680-labeled macrophages was imaged by FMT epifluorescence at multiple times post transfer (A). Quantification of paw swelling and macrophage recruitment (B) were assessed by engineer's micrometer measurement of paw thickness and quantification of paw epifluorescence, respectively. Epifluorescence data was thresholded to minimize non-specific background signal in negative control paws to below 20 counts/energy.

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