A Macrophage-Specific Fluorescent Probe for Intraoperative Lymph Node Staging

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Abstract

Successful identification of nodal metastases in patients with cancer is crucial to prescribe suitable treatment regimens that can improve recurrence-free survival. Although some new imaging technologies for nodal staging have been developed, such as nanoparticle-enhanced MRI and quantum-dot–based fluorescence imaging, sound technologies for intraoperative differentiation of metastatic and inflamed lymph nodes remain lacking. In this study, we illustrate the feasibility of using a macrophage-specific fluorescent probe (MFP) to visualize sentinel lymph nodes during surgery, highlighting abnormalities related to inflammation and tumor infiltration with signal enhancement and reduction methods using this technology. MFP was identified by high-throughput screening of fluorescent small-molecule libraries synthesized with a diversity-oriented approach. It selectively visualized monocyte and macrophage cell populations in vitro, by live-cell imaging and flow cytometry, as well as in vivo, for imaging-guided surgery. Collectively, this study provides preclinical proof of concept for an intraoperative imaging platform to accurately assess lymph node status, eliminating the need for invasive nodal dissections that can contribute to complications of cancer therapy. Cancer Res; 74(1); 44–55. ©2013 AACR

Introduction

Intraoperative/interventional fluorescence imaging is an evolving field of imaging science that offers new ways to highlight abnormalities in patients in real-time (1–3). Common radiologic modalities such as MRI and positron emission tomography (PET) are not suitable for intraoperative/interventional applications due to their high levels of irradiation and lack of integration in the operating room. In contrast, optical imaging is a natural approach in a variety of interventional procedures because it closely relates to the doctor’s vision during operation, and provides high spatiotemporal resolution and sensitivity. There have been vibrant developments of new fluorescent-probe technologies for labeling many cellular and subcellular processes intraoperatively (4–6). This imposed contrast is now captured with an increasing number of new optical imaging systems (7) such as multispectral reflectance fluorescence imaging (8, 9) and dual-modality catheter (10). Such advances have already started to impact clinical practice by providing real-time feedback on disease distribution. Recently, the first-in-human use of intraoperative tumor-specific fluorescence imaging with a folate receptor-α–targeted fluorescent agent was reported (11). In addition, endoscopic identification of dysplasia using fluorescent lectins (12) or targeted heptapeptides (13) has been demonstrated in a pilot study involving patients. Accurate real-time imaging with specific fluorescent agents is now shifting the paradigm for the operating suite from blind surgery based on anatomic contrast and tactile information to elaborate imaging-guided surgery offering real-time pathology.

One elegant example of intraoperative fluorescence imaging is sentinel lymph node (SLN) biopsy (SLNB) using visual guidance with fluorescent probes. The nodal status of cancer is an important prognostic indicator for patients diagnosed with many forms of solid cancers (14–18). The SLN concept states that, if the first lymph node to receive lymphatic drainage from a tumor site does not contain tumor cells, the tumor is not likely to have metastasized to the lymphatic system. Since the initial introduction of SLNB in 1992 (19), it has been a gold-standard for cancer management and has been widely used in many countries (20). The identification of the SLN is typically achieved by the use of radionuclide colloids (Technetium-99m) with a γ-camera (21, 22) and/or the injection of blue dye (isosulfan blue, methylene blue; refs. 23, 24), subcutaneously in the vicinity of the tumor tissue at the time of surgery. However, radiolabeled colloids have limited precision and show a slow uptake into the lymphatic system of up to 3 hours due to their bulky size. Blue chromophores rapidly attenuate the light penetration depth and show a high rate of false-negative results because the small dye molecules can readily diffuse through the true SLN to the second- and the third-tier nodes. Therefore, quantum dots (QDs; ref. 25) or fluorescent dyes such as indocyanine green (26, 27) and IRDye800 (28) have emerged as promising alternatives for sensitive and more accurate localization of SLNs. QDs have

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doii: 10.1158/0008-5472.CAN-13-2129

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exhibited rapid uptakes into the lymphatic system and remain in the SLN owing to their innate lymphotropic properties with hydrodynamic diameters of 10 to 50 nm.

Although intraoperative fluorescence imaging approaches, especially those using fluorescent nanoscale agents (29, 30), enable rapid uptake into the lymphatic system and effective mapping of the SLN, invasive surgical dissection of the node, followed by time-consuming examination by a pathologist, is still required (31–33). If the necessity for a painful second surgery after pathologic examination of the SLN following the removal of the primary cancer and SLNB or a delay in the surgical procedure due to intraoperative histology of the SLN during the surgery is to be avoided, real-time visual assessment of metastatic lymph nodes is needed to guide the surgeon’s scalpel without any unwanted interference with standard surgical procedures. Recently, a new superparamagnetic iron-oxide nanoparticle (SPION) was developed as an MRI contrast agent to identify tumor-infiltrated lymph nodes in patients with prostate cancer (34). Contrast-enhanced MRI correctly identified all patients with nodal metastases in whom nodal infiltration of macrophages that had engulfed the superparamagnetic particles highlighted metastatic regions devoid of signal, and a node-by-node analysis had a significantly higher sensitivity than conventional MRI. This preoperative imaging technology has implicated the possibility of using a synthetic fluorochrome with macrophage-specific binding affinity to provide effective intraoperative guidance on nodal involvement in cancer. Therefore, such a macrophage-specific fluorescence imaging modality may be very helpful to differentiate tumor-induced and inflammation-induced lymph node enlargement based on reduced and enhanced signals.

Recently, small-molecule fluorescent probes, in conjunction with significant advances in in vivo microscopy and interventional clinical imaging, have gained particular attention for reliable in vivo labeling (35, 36). Completely defined synthetic vital dyes have considerable potential to rapidly label cells, subcellular compartments, and molecular effectors in living objects due to their diverse biologic properties caused by skeletal and stereochemical complexities (37, 38). In this study, we report the development of a macrophage-specific fluorescent probe (MFP) using a diversity-oriented synthesis (39, 40) for intraoperative lymph node staging. A boron-dipyrromethene (BODIPY)–based MFP was discovered in a screening of 1,153 tailor-made, organic, fluorescent, small molecules (41, 42) against different blood-cell lines, including macrophages. Here, we show the capability of MFP to target monocytes/macrophages with high selectivity for live-cell microscopy and flow cytometry. We then demonstrate that the injection of MFP allows sensitive in vivo epifluorescence imaging of the SLN within minutes because of active binding macrophages, a main component of lymph nodes, and minimizing diffusion. Lymph node-mapping data in the inflammation and the metastasis mouse models showcase the potential of MFP for accurately assessing nodal status as a possible alternative to SPION-MRI in surgical guide imaging. Such an intraoperative macrophage-specific imaging with a small-molecule fluorescent probe strategy may be beneficial for improving surgical lymph-node staging and for making the clinical management of cancer more effective.

Materials and Methods

Cell preparation and differentiation

MOLT-4, HL-60, and U-937 cells were obtained from the American Type Culture Collection (ATCC) and were maintained according to ATCC protocols using RPMI1640 medium with 10% FBS, 1× GlutaMAX, 1× NEAA, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). For differentiation into macrophages, the nonadherent monocyte-like U-937 cells (2 × 10^6/mL) were treated with 20% LPS (100 ng/mL) for 7 days to induce differentiation. Following incubation, all cells were washed with PBS to remove any probe left in the solution and to optimize the background signal. U-937-DMs were dissociated and collected using 0.25% trypsin-EDTA. Following incubation, all cells were treated with 0.25% trypsin-EDTA for 1 hour before analysis by flow cytometry.

Chemical libraries and high-throughput screen

The combinatorial libraries of 1,153 fluorescent small molecules from diversity-oriented synthesis were screened against lymphocytes (MOLT-4) and monocytes/macrophages (HL-60, U-937, U-937-DM), which were plated onto 384-well plates at a density of 7,000 cells/well in a culture medium. The fluorescent compounds were altered at a final concentration of 0.5 or 1 μmol/L in a volume of 100 μL per well containing 0.1% dimethyl sulfoxide (DMSO; v/v). After compound incubation for 1 hour at 37°C, the cells were counterstained with Hoechst33342 and imaged using an automated high-throughput imager (ImageXpress System, Molecular Devices). The hits that stained monocytes/macrophages more brightly than lymphocytes were selected on the basis of the imaging performance and the intensity histogram with the aid of high-content imaging analysis software (MetaXpress, Molecular Devices).

Synthesis and characterization of MFP

A detailed description of the synthesis of the MFP can be found in the Supplementary Materials. The synthesized MFP was characterized using liquid chromatography–mass spectrometry (LC–MS) as well as both 1H and 13C nuclear magnetic resonance (Supplementary Figs. S2–S4).

Cell line imaging and flow cytometry

The cells were incubated with 0.8 μmol/L and 50 nmol/L MFP for microscopy and flow cytometry, respectively, in the culture medium for 1 hour at 37°C in a 5% CO2 atmosphere. Following incubation, all cells were washed with PBS to remove any probe left in the solution and to optimize the background signal. U-937-DMs were dissociated and collected using 0.25% trypsin-EDTA.
trypsin–EDTA for flow cytometry. Imaging experiments were performed on a LSM5-DUO confocal microscope (Carl Zeiss). All MFP fluorescence images were obtained with identical measurement parameters and contrasts. Flow cytometry was performed using a LSR-Fortessa cytometer (BD), and data were analyzed using FlowJo software (Tree Star).

Mice
A total of 20 Balb/c nude mice from the Biological Resource Center (BRC; Biomedical Sciences Institute, A’STAR) were used for the experiments, and their use was approved by the BRC Institutional Animal Care and Use Committee. For lymph node metastasis-evaluation experiments, human lung carcinoma NCI-H460 cells (ATCC, 5 × 10⁶) were implanted with Matrigel (BD) on the left dorsal flank of athymic mice. Two to 3 weeks after injection, in vivo imaging experiments were performed. Lipopolysaccharide (LPS)-induced inflammation models were generated by intra-lymph node injection of LPS (5 mg/kg; Sigma) 2 days before the imaging experiments.

In vivo fluorescence imaging of lymph node
Mice were anesthetized by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). The body temperature of the mice was kept constant at 37°C during all procedures. The MFPs [50 μmol/L in 50 μL of saline containing 1% of poly(ethylene glycol)4600(PEG) and 0.1% of Tween 20] were intradermally injected either in the left forepaw, for normal and inflammation model imaging, or in both the forepaws for tumor model imaging. A detailed description of reproducible injection technique can be found in the Supplementary Materials. An OV100 small animal imaging system (Olympus) was used to take fluorescence images of the axillary lymph nodes after injection. In vivo imaging data was analyzed with Matlab software (MathWorks). The SLN’s target-to-background ratio (TBR) was determined as the ratio of the mean intensity calculated on the identified lymph node region divided by the one calculated in a similar area of surrounding fat and muscle tissue. The regions of interest (ROI) were manually chosen over the entire lymph node, as well as in areas of surrounding tissue and areas outside the mouse’s body. The intensity measurements were repeated 3 times and averaged, and the mean intensity and SD were used for the TBR calculations. Data are presented as mean ± SD. Group comparisons were done by means of the unpaired Student t test.

Immunohistochemistry
After in vivo fluorescence imaging, the mouse was euthanized and the axillary lymph node was dissected. After immediate ex vivo fluorescence imaging, the lymph node was embedded in tissue-freezing medium (Triangle Biomedical Sciences), frozen, and consecutively cryo-sectioned in 8-μm segments. The tissue sections were rinsed with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. After an additional series of washes with PBS, the tissue sections were cleared with 3% sodium deoxycholate solution for 2 hours at room temperature, blocked with 20% normal goat serum in 1% BSA-PBS for 2h at 37°C, incubated with the primary antibodies at 4°C overnight (>17 hours), diluted in 1% BSA–PBS, rinsed 3 times with PBS, and incubated with secondary antibodies at 4°C for 2 hours. Then, the slides were washed with PBS several times, counterstained with Hoechst33342, and mounted with ProLong Gold antifade reagent (Life Technologies). The images were captured with an Eclipse Ti-E microscope (Nikon).

Results
Screening identifies the unique fluorescent small molecule, MFP
Several classes of fluorescent small molecules (MW < 1,000 Da) were synthesized based on a diversity-oriented approach in order to combine the properties of conjugatability and biocompatibility. Libraries comprising 1,153 compounds were screened against different human blood cell lines, that is, MOLT-4, HL-60, U-937, and U-937-derived macrophages (U-937-DM; Supplementary Fig. S1). We were particularly interested in the selection of fluorochromes that could discriminate between lymphoid (MOLT-4) and myeloid cells (HL-60, U-937, U-937-DM). As a final hit, an MFP was discovered and showed the most preferential staining for all myeloid lineage cells, compared with lymphoid cells, based on imaging performance and intensity histogram analyses (Supplementary Fig. S1).

For further studies, we scaled up the synthesis of MFP and performed characterization as summarized in Supplementary Figs. S2–S4. MFP (λex/λem = 562/589 nm; Supplementary Fig. S5) has a molecular weight of 505.75 Da, and displays excellent photophysical properties with a high fluorescent quantum yield (0.15) and a high extinction coefficient (7.61 × 10⁴ mol/L per centimeter). Fig. 1A shows an apparent preference of MFP for primitive human myeloid cells (HL-60), monocytes (U-937), and macrophages (PMA-stimulated U-937 cells), as well as murine macrophages (RAW 264.7), when using confocal microscopy. In addition, a flow cytometric analysis confirmed the selectivity of the probe (Fig. 1B). The quantification shows a greater than 3-fold difference in intensity signal between myeloid and lymphoid cells for both microscopy and flow cytometry (Fig. 1C and D). Differentiation of U-937-DMs was confirmed with differentiation markers (CD68, CD36) by immunocytofluorescence and flow cytometry (Supplementary Fig. S6). MFP proved to be robust for live-cell imaging by probing for monocytes/macrophages selectively at various concentrations (100–800 nmol/L) and incubation times (1–4 hours; Supplementary Fig. S7). In addition, structural selectivity was tested with several negative analogs of MFP (Supplementary Fig. S13). Even at a high concentration of MFP (25 μmol/L) for 24 hours, the adverse effects of the probe on cell viability were minimal (Supplementary Fig. S8).

White blood cell phenotyping by MFP
We next tested peripheral blood taken from healthy human donors to further evaluate the feasibility of using MFP for white-blood-cell phenotyping. Primary human T lymphocytes, granulocytes, monocytes, and monocyte-derived macrophages (Supplementary Figs. S9 and S10) with macrophage colony-stimulating factor from three independent donors were all probed with MFP in duplicates. Fig. 2A depicts representative
flourescence images demonstrating MFP as a compound preferred for primary monocytes and macrophages. All MFP fluorescence images had identical exposure times and same overall contrast settings to measure relative intensity difference. Phenotypic characterization of nucleated blood cells in flow cytometry was feasible when employing MFP in conjunction with minimally manipulated whole-blood samples (Fig. 2B and 2C) although competition for compound uptake was increased, thus specificity for monocytes was reduced in whole blood environment compared with separated blood cell test (Fig. 2A). As shown in Fig. 2B and D, monocytes, granulocytes and lymphocytes—the three main components—can be represented as MFP<sup>bright</sup>, MFP<sup>intermediate</sup>, MFP<sup>dim</sup>, respectively, on the flow dot plot and histogram. The quantification shows 1.73- and 13.2-fold difference in the fluorescence signal for monocytes as compared with those for granulocytes and lymphocytes, respectively (Fig. 2C).

To showcase the capability of MFP to isolate each of these cell populations, we performed fluorescence-activated cell sorting (FACS) for human leukocytes in MFP<sup>bright</sup>, MFP<sup>intermediate</sup>, MFP<sup>dim</sup> populations. Immunofluorescence staining was performed with cytospin preparations for each population. Figure 2E shows a high expression of the monocyte marker CD14 in MFP<sup>bright</sup> cells, a high expression of the granulocyte marker CD66b in MFP<sup>intermediate</sup> cells, and a high expression of the T lymphocyte marker CD3e and the B lymphocyte marker CD19 in MFP<sup>dim</sup> cells, indicative of successful isolation of the three main components of peripheral blood.

MFP SLN mapping

Due to the specific macrophage-targeting ability of MFP, we explored whether MFP could be used for lymphatic mapping because macrophages are the major cell type in lymph nodes. After an intradermal injection in the lymphatic-rich front paw of a mouse, MFPs entered the lymphatic collecting vessels and migrated within minutes to an axillary node that could be detected by epifluorescence imaging (Fig. 3A and B). The fluorescent deposits became progressively denser, making the draining lymph node perceptible, and remained fixed in the node for hours, with minimum diffusion to the next node. Owing to the outstanding photophysical properties of the MFPs, we could follow lymphatic flow toward the regional node from the injected forepaw in real time with high sensitivity and then dissect the node following quick identification of its position (Fig. 3C and D), which mimics the SLNB.
procedure in cancer surgery. A magnified intraoperative image and an ex vivo image (Fig. 3C and D) showed that the fluorescence signal associated with the SLN, but not the adjacent fat tissue, indicated preferential accumulation of MFPs in the SLN.

**Lymph node inflammation imaging**

Having shown the ability to detect the SLN with MFPs, we proceeded to determine the ability of the MFP to distinguish a macrophage-rich, inflamed lymph node from a normal node. We first generated inflammation models (n = 3) by intra-lymph-node injection of LPS into the left axillary node. Two days after LPS injection, both the inflammation model and the control group received an intradermal injection of MFPs in the left forepaw. One hour after MFP injection, mice were imaged in vivo, and the node TBRs were calculated for the acquired fluorescence images. In the inflammation-induced mice injected with MFPs, intense signals in the axillary node were observed with in vivo fluorescence imaging, indicating elevated macrophage content (left in Fig. 4A and D). In contrast, the MFP-injected control mice demonstrated lower fluorescence signals (right, Fig. 4A and B). In the MFP-injected mice, the in vivo node TBR was more than 200% higher in the inflammation group than in the normal group (TBR: 5.8 ± 0.2 vs. 2.5 ± 1.5, P = 0.02; Fig. 4C).

In vivo imaging data were verified by immunohistofluorescence of lymph nodes harvested 1 hour after injection of the MFP. Using several markers specific for lymph-node–consisting cells, we were able to identify the characteristics of the MFP localization (Fig. 5). As shown in the first row in Fig. 5A and B, MFP fluorescence localized in the scattered large cells within the subcapsular sinus (SCS) and the medullary sinus—areas that are well-known locations for lymph node macrophages. MFP-binding cells located beneath the SCS floor were typically in contact with the lymph compartment that contains B cells in the underlying follicles (Fig. 5A and B, the second bottom row).
thus showing structural features of macrophages. Indeed, most MFP$^+$ cells expressed macrophage markers, including CD68, CD11b, and F4/80, showing selective macrophage targeting of MFP in the SCS and medulla (Fig. 5 and Supplementary Fig. S11). The MFP signal, however, did not colocalize with LYVE-1$^+$ lymphatic endothelial cells surrounding medullary sinuses, Gr-1$^+$ granulocytes and B220$^+$ follicular B cells. The higher content of MFP-binding cells stained with macrophage markers in an inflamed node (Fig. 5A) compared with that in a normal node (Fig. 5B) in accordance with in vivo fluorescence imaging.

**Detection of metastases in draining lymph node**

Recently, a SPION was developed as an MRI contrast agent to identify tumor-infiltrated lymph nodes and was successfully applied in clinics (34). This technology relies on an indirect identification of metastasis in which nodal infiltration of macrophages that have engulfed the magnetic particles highlights metastatic regions devoid of signals. On the basis of its highly specific targeting ability for macrophages, we hypothesized that MFP should enable in vivo assessment of lymphatic metastasis, thus allowing for an excellent changeover from SPION-MRI to intraoperative fluorescence imaging.

To test the hypothesis, we generated xenograft models of lung cancer ($n = 4$) by left dorsal injection of NCI-H460 cells to promote metastasis in the left axillary node. Two to 3 weeks after injection, in vivo imaging experiments were performed after intradermal injection of the MFP. Fig. 6 shows the diagnostic capability of the MFP to detect lymph node metastasis using a deficient fluorescence signal caused by the replacement of macrophages by disseminated tumor cells. Injecting the MFP into both forepaws produced a reduced signal in the ipsilateral axillary node, but an enhanced signal in the contralateral axillary node (Figs. 6A–6F). Intraoperative fluorescence imaging well delineated the precise location of the nodal metastasis. The contralateral node’s TBR with in vivo fluorescence images was 21.9 ± 2.3. In contrast, the ipsilateral node exhibited low signals (180%–200% decrease), with a node TBR of 10.6 ± 3.1 ($P = 0.001$) indicating the replacement of macrophages by tumor lesions. Moreover, the intraoperative TBR was more than 360% lower in the ipsilateral node than in the contralateral node (TBR: 6.8 ± 3.1 vs. 24.8 ± 2.0, $P = 0.00007$). Ex vivo fluorescence imaging after image-guided surgical resection of the respective lymph nodes confirmed the significant intensity difference between the ipsilateral and the contralateral lymph nodes, which were considered as macrometastatic and micro-/nonmetastatic nodes, respectively. The extensive tumor-cell infiltration in the
ipsilateral node was confirmed by the node’s enlarged size (Figs. 6C and F) and immunohistofluorescent identification with a tumor-specific HER2 antibody (Fig. 7).

The paw-directed injection of MFP in the tumor model resulted in rapid dispersion to the draining axillary node and selective lymph node macrophage labeling. Immunohistofluorescence of frozen lymph node serial sections (Fig. 7 and Supplementary Fig. S12) revealed a specificity of the MFP to the macrophages that was similar to the specificity obtained from the inflamed-node analysis. All of the MFPs bound to CD68⁺ or CD11b⁺ or F4/80⁺ macrophages, but not to LYVE-1⁺ endothelial cells, Gr-1⁺ granulocytes, CD11c⁺ dendritic cells, B220⁺ B lymphocytes or HER2⁺ cancer cells (Fig. 7 and Supplementary Fig. S12). As predicted with in vivo imaging, large macroscopic metastatic lesions with a high density of HER2⁺ cells were present in the left axillary node, whereas only microscopic infiltration of HER2⁺ tumor cells was detected in the right node.

Discussion

Recent technologic evolutions in optical imaging and the increasing list of fluorescent probes have driven the rapid progression of fluorescence imaging into interventional clinical procedures. Fluorescent guidance for SLNB has received particular attention due to the significance of lymph nodes as the first clinically observed site of most cancer metastasis. The most common approach for SLN visualization is with the use of nonspecific nanoscale fluorescent agents with hydrodynamic diameters of 10 to 50 nm, which are in the preferred range for lymphatic uptake. Although these techniques have improved surgical practice for sensitive and accurate identification of SLNs, lymph node staging for metastasis can only be determined by preoperative SPION-MRI or postoperative histopathologic analysis followed by an invasive lymphadenectomy. In this proof-of-concept study, we investigated the potential value of intraoperative macrophage-specific fluorescence imaging for pinpointing a suspicious lymph node to improve surgical outcomes for patients with cancer using a xenograft metastasis model.

An essential component of this technology is the development of MFPs through a high-throughput screen of synthetic vital-dyes prepared by small-molecule chemistry with a process compatible with good manufacturing practices. We showed that, in mice, MFPs injected in the interstitial space of the paw were readily drained via lymphatic vessels to downstream axillary lymph node. Once the MFP had entered
intranodal lymph conduits, it specifically binds strongly with the SCS and medullary macrophages, but not with any other node-constituting cells, thereby minimizing diffusion to the next node and highlighting nodal inflammation. In addition, we found that lymph node metastases can be accurately detected by a node-by-node comparison of MFP uptake. MFPs selectively bound in macrophages of normal or micrometastatic lymph nodes, increasing the nodal fluorescence signal intensity. Nodes that are completely replaced by tumor tissue lack uptake of MFPs and generally show a low fluorescence signal. MFP binding in lymph nodes is reduced in the context of tumor deposit, whereas ideally one might select a direct imaging agent that shows an increased signal from metastatic lesions compared to normal nodes. There is, however, a potential benefit to using an indirect contrast agent, such as an MFP for intraoperative lymph node imaging. The MFP is capable of detecting not only nodal metastases but also local inflammation. The capability of the MFP to differentiate between metastatic and inflammatory lymph nodes will provide important functional information for surgical guidance because inflammation- and tumor-induced lymph node...
enlargement can be misinterpreted, leading to invasive dissection of benign nodes (43). Moreover, macrophage-specific fluorescence imaging can be used in most cancer surgeries, regardless of specific tumor type, because displacement of nodal macrophages by tumor cells is a general phenomenon of most cancers (44). Together with these advantages, additional studies to establish standards of injection and image analysis can make this technique more attractive for clinical use. Especially, recent multispectral reflectance imaging (8) or optoacoustic technology (45) could be employed together for quantitative nodal staging by suppressing tissue-autofluorescence and correcting light-attenuation.

The lymph node is not only a clinically important site for cancer prognosis but also a valuable organ to illuminate the immune mechanism. In vivo live-cell imaging reveals lymph node cellular dynamics, and is becoming increasingly useful in the investigation of adaptive and innate immunity (46). Recently, lymph node macrophages were identified as crucial gatekeepers to prevent fatal viral invasion (47–49). Because of the specific and stable macrophage targeting ability, MFP-mediated live-cell imaging to monitor the behavior of lymph node macrophages will have certain benefits in the study of how they respond to pathogens in native physiologic settings. One interesting application of the lymph node is its use as an ectopic transplantation site for multiple tissues and organ regeneration (50). To make it realistic, the immune reactions within lymph nodes must be tested when healthy donor cells are injected. The MFP live-cell imaging technique can then be used to observe interactions of macrophages with engrafted cells inside lymph nodes to validate immunogenicity. Overall, live imaging of lymph node macrophages with an MFP can shed new light on the in vivo biology of the immune system, providing measurements of cellular interactions and response dynamics.

In summary, we present the targeted fluorescent agent MFP as a valuable research tool for in vivo behaviors of macrophages.
in lymph nodes, as well as a clinical navigation to aid surgeons in debulking efforts by providing real-time information on adenopathy related to inflammation and caused by infiltration of tumor cells. Intraoperative macrophage-specific fluorescence imaging offers a unique opportunity for in situ detection of lymph node metastases during cytoreductive surgery without the need for a preoperative lymph node biopsy or for intraoperative histology. Therefore, we consider this study to be of clinical relevance and anticipate it to have considerable influence on the development of a new surgical oncologic imaging platform for lymph node staging.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J.S. Yoo, Z.Y. Jow, Y.-T. Chang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Yoo, Z.Y. Jow
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Acknowledgments
The authors thank Drs. P.W. Kuchel and J. Kim for assistance with the human blood cell experiments, and H.S. Cheng for management of tumor mouse. The authors also thank Drs. C.L. Teoh and R.E. Ericksen for critical review, and Drs. K.-S. Soh, E. Kim, and M.H. Kim for scientific discussion. Microscopy data for immunohistochemistry were acquired and analyzed in the SBIC-Nikon Imaging Centre at Biopolis.

Grant Support
This study was financially supported by intramural funding from the A’STAR (Agency for Science, Technology and Research, Singapore) Biomedical Research Council and the Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2010-T2-2-030).

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Received July 29, 2013; revised October 30, 2013; accepted October 31, 2013; published OnlineFirst December 9, 2013.

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