



## Imaging of the interaction of cancer cells and the lymphatic system<sup>☆</sup>

Hop S. Tran Cao<sup>a,1</sup>, Michele McElroy<sup>a,1</sup>, Sharmeela Kaushal<sup>b,1</sup>,  
Robert M. Hoffman<sup>a,b,c,2</sup>, Michael Bouvet<sup>a,b,\*</sup>

<sup>a</sup> Department of Surgery, University of California, San Diego, CA, USA

<sup>b</sup> Moores Cancer Center, University of California, San Diego, CA, USA

<sup>c</sup> AntiCancer Inc., San Diego, CA, USA

### ARTICLE INFO

#### Article history:

Received 5 June 2010

Accepted 9 May 2011

Available online 28 June 2011

#### Keywords:

Antibody–fluorophore conjugate

Cancer cell

Lymphatic delivery

### ABSTRACT

A thorough understanding of the lymphatic system and its interaction with cancer cells is crucial to our ability to fight cancer metastasis. Efforts to study the lymphatic system had previously been limited by the inability to visualize the lymphatic system *in vivo* in real time. Fluorescence imaging can address these limitations and allow for visualization of lymphatic delivery and trafficking of cancer cells and potentially therapeutic agents as well. Here, we review recent articles in which antibody–fluorophore conjugates are used to label the lymphatic network and fluorescent proteins to label cancer cells in the evaluation of lymphatic delivery and imaging.

© 2011 Elsevier B.V. All rights reserved.

### Contents

1. Introduction . . . . .	886
2. Antibody-based lymphatic imaging . . . . .	886
3. Antibody inhibition of lymphatic proliferation in cancer. . . . .	887
4. Imaging of lymphatic cancer cell trafficking <i>in vivo</i> . . . . .	888
5. Conclusions . . . . .	888
Acknowledgements . . . . .	889
References . . . . .	889

## 1. Introduction

The lymphatic system plays a critical role in tumor progression and is often an early route in cancer metastasis. Lymphatic involvement is a key component of cancer staging and adversely affects overall prognosis. The dynamics by which cancer cells enter the lymphatic system, travel to distant sites, and eventually establish metastatic foci,

remain poorly understood. However, approaches to understanding this process have grown over the past few decades to address two main purposes: first, to image lymphatic drainage in order to identify lymph nodes that may be involved in the metastatic process and thus assist clinicians in the staging of cancer, and second, to potentially deliver therapeutics via the lymphatic system to prevent or curtail lymphatic spread of cancer cells. These objectives may be achieved partly by the utilization of specific antibodies, to lymphatic components or to cancer cells, conjugated to fluorophores, that allow imaging of the lymphatic system in a novel and accurate way.

## 2. Antibody-based lymphatic imaging

Identification of the sentinel node has long been understood to be important in the staging of many cancers, including breast cancer and melanoma [1]. Agents used for sentinel node detection include blue dyes, such as isosulfan blue dye, and colloidal radiotracers, mainly Technetium-99 sulfur colloid [2,3]. Both of these agents are locally

<sup>☆</sup> This review is part of the *Advanced Drug Delivery Reviews* theme issue on “Lymphatic Drug Delivery: Therapy, Imaging and Nanotechnology”.

\* Corresponding author at: 3855 Health Sciences Drive #0987, La Jolla, CA 92093-0987, USA. Tel.: +1 858 822 6191; fax: +1 858 822 6192.

E-mail addresses: [htrancao@ucsd.edu](mailto:htrancao@ucsd.edu) (H.S.T. Cao), [mkmcelroy@ucsd.edu](mailto:mkmcelroy@ucsd.edu) (M. McElroy), [skaushal@ucsd.edu](mailto:skaushal@ucsd.edu) (S. Kaushal), [all@anticancer.com](mailto:all@anticancer.com) (R.M. Hoffman), [mbouvet@ucsd.edu](mailto:mbouvet@ucsd.edu) (M. Bouvet).

<sup>1</sup> 3855 Health Sciences Drive, La Jolla, CA 92093, USA. Tel.: +1 858 822 4176; fax: +1 858 822 4181.

<sup>2</sup> 7917 Ostrow St, San Diego, CA 92111-3604, USA. Tel.: +1 858 654 2555; fax: +1 858 268 4175.

injected intradermally, rely on the natural uptake of molecules in the interstitial spaces by lymphatic vessels, and subsequently travel to the first lymph node draining this particular area. However, since they lack specificity, they tend to further extend to other lymph nodes as well. A newer agent, named Lymphoseek, was developed specifically to map the sentinel node by lymphoscintigraphy [4]. Lymphoseek is a molecule consisting of a dextran backbone to which several units of Diethylenetriaminepentaacetic Acid (DTPA) and mannose are attached. The DTPA units allow binding of Technetium-99m to allow for detection with the use of a gamma probe, and the mannose units bind to receptors found on the surface of macrophages that tend to accumulate in the sentinel lymph node. Thus, Lymphoseek is a radiolabeled reporter that has the advantage of being specific for the sentinel node. However, as all currently available tracers, Lymphoseek offers no information on the presence of cancer cells in these sentinel nodes.

The use of specific antibodies for detection of lymphatic metastasis was first studied by Weinstein et al. [5,6]. The authors established that monoclonal antibodies (Mab) injected intradermally entered lymphatics, traveled to regional lymph nodes, and accumulated in metastatic lymph nodes. They detected antibodies by radio-labeling. This immunolymphoscintigraphy model was the basis of several subsequent studies on the process of lymphatic delivery of substances and tumor cell trafficking.

Currently, efforts to more clearly define this process are enhanced by the development of fluorescence imaging. For example, Sharma and colleagues were able to quantitate lymphatic flow with the use of a near-infrared (NIR) fluorophore injected intradermally [7]. Fluorescence imaging has advantages over previous tracers such as blue dyes and colloid radiotracers in that it allows live, real-time imaging of the lymphatic system, and permits evaluation of lymphatic function as well. Specific fluorescent antibodies enable imaging of the interaction between the lymphatic system and cancer cells.

Although other agents are used to perform lymphatic mapping, they are non-specific and require local injection at the tumor site. Licha et al. sought to evaluate whether systemic, intravenous injection of a fluorophore-conjugated antibody directed against glycoproteins found on the luminal side of specialized high endothelial venules (HEV) in lymph nodes, such as the MECA-79 antibody, would allow recirculation of this antibody to peripheral lymph nodes [8]. After confirming its specificity via microautoradiography and organ distribution analysis, the authors conjugated MECA-79 antibody with a near-IR cyanine fluorescent dye with absorption and fluorescence peaks at 750 nm and 771 nm, respectively. A second conjugate with near identical properties was synthesized that contained a control antibody instead. Both conjugates were injected intravenously via the tail vein at equal doses in nude mice. Fluorescence images were then obtained of both groups of mice at multiple time points. The authors found that the MECA-79 antibody-fluorophore conjugate bound specifically to lymph nodes as early as 10 minutes after injection, with maximal contrast being achieved at 24 hours post-injection. By comparison, the control antibody-fluorophore conjugate failed to concentrate in lymph nodes. Lymph nodes were also collected from animals sacrificed 24 minutes after injection of the MECA-79 antibody cyanine-dye-conjugate. The tissues were semiquantitatively analyzed for fluorescence signal intensity, and as expected, displayed higher fluorescence intensities than nodes collected from mice injected with the control antibody dye conjugate. This study identifies MECA-79 antibody as an appropriate imaging probe for fluorescence imaging of lymph nodes.

While MECA-79 antibody allows homing of the contrast agents into lymph nodes, it is not directed against cancer cells, and is thus non-specific. To address this issue, Sampath and colleagues recently developed dual-labeled trastuzumab, a monoclonal antibody specific for the human epidermal growth factor receptor, HER2, present on the surface of up to 30% of breast cancer [9]. HER2 is associated with a

poor prognosis, and trastuzumab has been shown to have therapeutic value in the treatment of HER2-positive breast cancer [10]. In this study, the authors conjugated the antibody to both a nuclear radiotracer and a near-IR fluorophore, IRDye 800 [9]. The specificity of this conjugate was tested *in vivo* by both nuclear and fluorescence imaging of nude mice bearing HER2-overexpressing human breast cancer. The conjugate was injected intravenously, and aggregated most strongly to the tumor region, as demonstrated by both imaging modalities. When scintigraphy and fluorescence images were compared, the signal-to-noise ratio was found to be significantly greater in fluorescence imaging. The results of this study demonstrated that cancer-specific antibodies can target cancer cells when delivered intravenously.

In the second part of the study, the potential of using antibody-based optical imaging for lymph node imaging was determined. The conjugate was injected intradermally, and exhibited a similar pattern of lymphatic uptake and drainage as with the fluorophore alone, albeit at different accumulation and clearance rates. However, the mice in which this experiment was performed did not have tumors, so that the use of this anti-HER2 antibody was non-specific. A similar experiment in a HER2-overexpressing-tumor-bearing mouse could determine if the conjugate could target metastatic lymph nodes.

### 3. Antibody inhibition of lymphatic proliferation in cancer

Vascular endothelial growth factor (VEGF)-C expression has previously been shown to be associated with lymphangiogenesis and lymphatic metastasis via its interaction with both VEGF receptors 2 and 3 (VEGFR-2 and VEGFR-3) [11,12]. Hoshida et al. carried out a set of elegant experiments designed to study the effects of VEGF-C on lymphatic development, morphology, and function, combining fluorescence lymphangiography and VEGFR-2 and VEGFR-3 specific antibodies [13]. In this study, fibrosarcoma and melanoma cells were stably engineered to over-express VEGF-C and transduced to express green fluorescent protein (GFP). The lymphatic vessels in the ear of nude mice were delineated and studied with both blue dye and fluorescence lymphangiography. Next, VEGF-C overexpressing fibrosarcoma and melanoma cells were implanted at the tip of the ear, and a number of parameters were measured. First, the authors demonstrated that these cells were associated with an increased incidence of lymphatic metastasis when compared with mock-transduced control cancer cells. This is likely partly due to VEGF-C-induced enlargement of peritumor lymphatic vessels, which were measured with epifluorescence and multiphoton laser scanning intravital microscopy. This finding was confirmed by histochemical staining with LYVE-1 and podoplanin antibodies. Both are specific markers of lymphatic endothelial cells and are responsible for an overall increased lymph flow rate. Furthermore, VEGF-C appears to create abnormal lymph flow patterns.

Using the same fluorescence imaging modality, the authors were able to acquire static images of GFP-expressing cells shed from the tumors into the peritumor lymphatics and eventually into the draining lymph node. Their finding revealed a 200-fold increase in tumor cell accumulation in the lymph node in tumors overexpressing VEGF-C compared to control tumors 10 days after tumor implantation. No differences in migratory, proliferative, or survival properties among the cancer cells themselves were observed.

The roles of VEGFR-2 and VEGFR-3 were further investigated in this study with the use of monoclonal antibodies. Blockage of VEGFR-3 reduced lymphatic hyperplasia and tumor cell delivery to the lymphatic system, but failed to inhibit growth of cancer cells seeded in the lymph node. Blockade of VEGFR-2, on the other hand, successfully inhibited growth of both primary tumors and lymph node metastases.

#### 4. Imaging of lymphatic cancer cell trafficking in vivo

The dynamic processes by which cancer cells invade the lymphatic system and travel within the lymphatic vessels to the draining lymph node and between nodes was previously poorly understood. We have developed real-time imaging of cancer cell trafficking in lymphatic vessels. Cancer cells dually-labeled with GFP in the nucleus and RFP in the cytoplasm or singly-labeled with GFP or RFP only were injected into the inguinal lymph node of nude mice. The labeled cancer cells trafficked through lymphatic vessels where they were imaged via a skin flap in real time at the cellular level until they entered the axillary lymph node. The bright fluorescence of the cancer cells and the real-time microscopic imaging capability of the Olympus OV100 small-animal imaging system enabled imaging of the trafficking cancer cells in the lymphatics in real time. Using this imaging strategy, two different cancer cell lines, one expressing GFP and the other expressing RFP, were simultaneously injected in the inguinal lymph node. Fluorescence imaging readily distinguished the two color-coded cell lines and their different abilities to survive in the lymphatic system. Using this imaging technology, we also investigated the role of pressure on tumor-cell shedding into lymphatic vessels. Pressure was generated by placing 25- and 250-g weights for 10 s on the bottom surface of a tumor-bearing footpad [14]. Single cells, and emboli shed from the footpad tumor were easily distinguished with the labeled cells and OV100 imaging system. Increasing pressure on the tumor increased the numbers of shed cells, and emboli. Pressure also deformed the shed emboli, increasing their maximum major axis. Imaging lymphatic trafficking of cancer cells can reveal critical steps of lymph node metastasis.

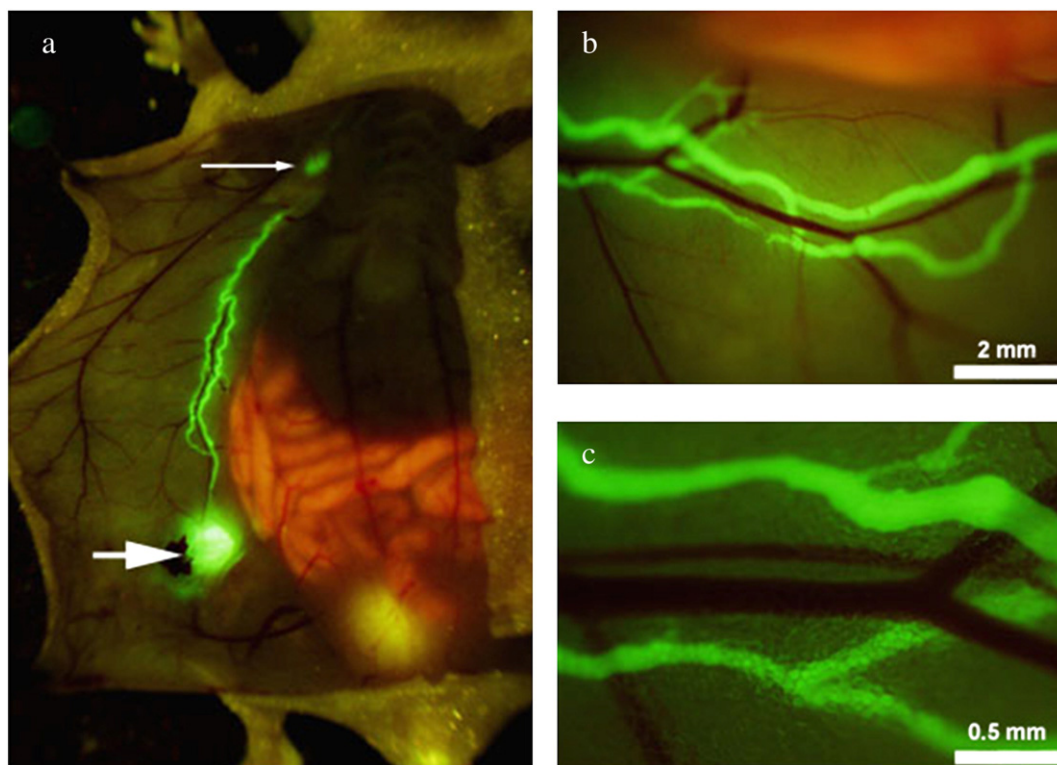
Our laboratory has also published a study that combines the fluorophore-conjugated lymphatic-specific antibody LYVE-1 with fluorescent-protein-labeled cancer cells to achieve in vivo color-coded imaging of tumor cell trafficking in lymphatic vessels and lymph nodes [15]. LYVE-1 is a hyaluronic acid receptor that is specific

for lymphatic endothelial cells. Anti-LYVE-1 antibodies had previously been used for immunohistochemical staining of lymphatic vessels. In our study, anti-LYVE-1 antibodies were conjugated with AlexaFluor 488 to image lymphatic vessels. A skin flap in a nude mouse was opened to expose the inguinal and axillary lymph nodes and connecting lymphatic vessels. The conjugate was then directly injected into the inguinal lymph node, allowing strong, distinct, and long-lasting visualization of the draining inguinal lymphatics as well as the afferent and axillary lymphatics (Fig. 1). The signal persisted up to 48 hours and was strongest at 4 hours. In comparison, control conjugated IgG or FITC-dextran administration to the inguinal lymph node had much less durable signals, with no signal visible at 12 hours post-injection. Specificity of anti-LYVE-1-antibody was verified *ex vivo* by fluorescence microscopy.

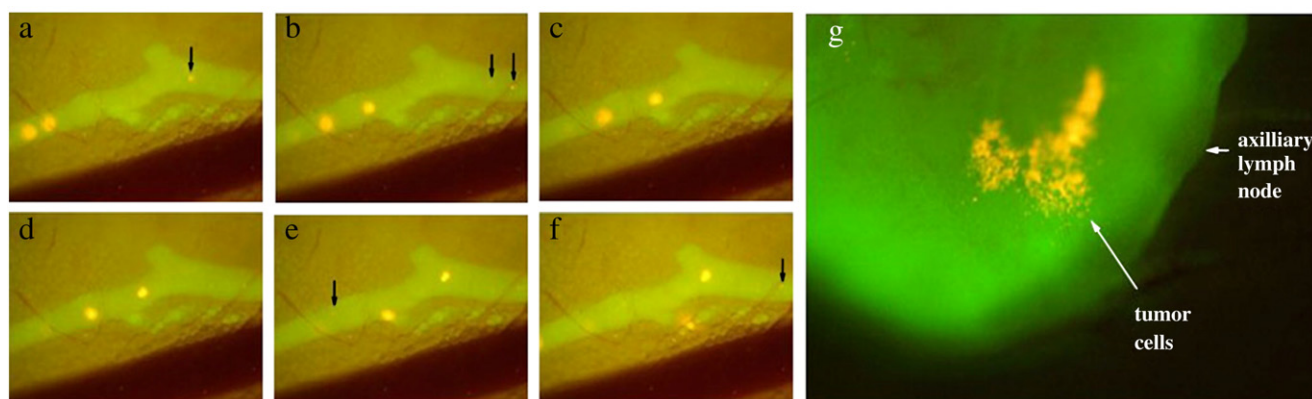
We next injected RFP-expressing XPA-1 pancreatic cancer cells into the inguinal lymph node and surrounding tissues. Fluorescence video imaging allowed real-time visualization of tumor cell trafficking through the lymphatic system. The contrast of RFP-expressing cancer cells on the bright green background of the lymphatic system afforded by AlexaFluor 488 enabled imaging of the cancer cells in real time as they traveled from the inguinal node to the axillary node (Fig. 2). Our study was the first to demonstrate in vivo cancer cell trafficking in fluorescence lymphangiography [15]. This powerful tool may offer significant advantages in future studies of the interaction between the lymphatic system and cancer cells.

#### 5. Conclusions

The unique interaction of cancer cells with the lymphatic network is a complicated process that includes the involvement of lymphatic drainage and metastasis. Our understanding of this process has been enhanced with the use of fluorescence imaging. This review makes demonstrates that antibody-fluorophore conjugates and fluorescent-



**Fig. 1.** *In vivo* fluorescence imaging of the ventral skin lymphatic system in a skin flap mouse model. AlexaFluor-conjugated anti-LYVE-1 antibody was injected into the inguinal lymph node (a, large arrow). Under fluorescence imaging, the inguinal lymph node, afferent lymphatics, and axillary lymph node (a, small arrow) display a strong, durable fluorescence signal that did not stain adjacent blood vessels (b, c).



**Fig. 2.** Time-sequential images of the same area of abdominal wall lymphatics (a–f) clearly show RFP-expressing XPA-1 human pancreatic cancer cells travel through the fluorescently-labeled lymphatic vessel. These cells could be seen accumulating in the axillary lymph node (g). The lymphatic system was highlighted by administration of AlexaFluor-conjugated anti-LYVE-1 antibody.

protein-labeled cancer cells can play an important role in understanding the interaction of cancer cells and the lymphatic system. The review has also demonstrated that MECA-79-antibody-fluorophore conjugates can identify lymph nodes; trastuzumab-fluorophore conjugates can localize specifically to HER2-expressing cancer cells; VEGFR-2 and VEGFR-3 antibodies can inhibit lymphatic hyperplasia and cancer cell seeding into the lymphatic system; and anti-LYVE-1-fluorophore conjugates can clearly delineate the lymphatic system and allow in vivo visualization of RFP-expressing cancer cells trafficking in lymphatic channels. The use of dual-photon imaging and red-shifted fluorophores can increase the depth capabilities of fluorescence imaging, allowing deeper non-invasive imaging of the interaction of cancer cells and the lymphatic system. The combination of these technologies with target-specific antibodies should provide us with an ever-growing understanding of the critical interplay between cancer cells and the lymphatic system.

#### Acknowledgements

This work was supported in part by grants from the National Cancer Institute CA142669 and CA132971 (to M.B. and AntiCancer, Inc) and T32 training grant CA121938 (to H.S.T.C. and M.M.).

#### References

- [1] M. Torabi, S.L. Aquino, M.G. Harisinghani, Current concepts in lymph node imaging, *J. Nucl. Med.* 45 (2004) 1509–1518.
- [2] A.J. Cochran, R. Essner, D.M. Rose, E.C. Glass, Principles of sentinel lymph node identification: background and clinical implications, *Langenbecks Arch. Surg.* 385 (2000) 252–260.
- [3] G. Mariani, L. Moresco, G. Viale, G. Villa, M. Bagnasco, G. Canavese, J. Buscombe, H.W. Strauss, G. Paganelli, Radioguided sentinel lymph node biopsy in breast cancer surgery, *J. Nucl. Med.* 42 (2001) 1198–1215.
- [4] A.M. Wallace, C.K. Hoh, D.R. Vera, D.D. Darrah, G. Schulteis, Lymphoseek: a molecular radiopharmaceutical for sentinel node detection, *Ann. Surg. Onc.* 10 (2003) 531–538.
- [5] J.N. Weinstein, R.J. Parker, A.M. Keenan, S.K. Dower, H.C. Morse III, S.M. Sieber, Monoclonal antibodies in the lymphatics: toward the diagnosis and therapy of tumor metastases, *Science* 218 (1982) 1334–1337.
- [6] J.N. Weinstein, M.A. Stellar, A.M. Keenan, D.G. Covell, M.E. Key, S.M. Sieber, R.K. Oldham, K.M. Hwang, R.J. Parker, Monoclonal antibodies in the lymphatics: selective delivery to lymph node metastases of a solid tumor, *Science* 222 (1983) 423–426.
- [7] R. Sharma, W. Wang, J.C. Rasmussen, A. Joshi, J.P. Houston, K.E. Adams, A. Cameron, S. Ke, S. Kwon, M.E. Mawad, E.M. Sevick-Muraca, Quantitative imaging of lymph function, *Am. J. Physiol. Heart Circ. Physiol.* 292 (2007) H3109–H3118.
- [8] K. Licha, N. Debus, S. Emig-Vollmer, B. Hofmann, M. Hasbach, D. Stibenz, S. Sydow, M. Schirner, B. Ebert, D. Petzelt, C. Bührer, W. Semmler, R. Tauber, Optical molecular imaging of lymph nodes using a targeted vascular contrast agent, *J. Biomed. Opt.* 10 (2005) 41205.
- [9] L. Sampath, W. Wang, E.M. Sevick-Muraca, Near infrared fluorescent optical imaging for nodal staging, *J. Biomed. Opt.* 13 (2008) 041312.
- [10] C.H. Yeon, M.D. Pegram, Anti-erbB-2 antibody trastuzumab in the treatment of HER2-amplified breast cancer, *Invest. New Drugs* 23 (2005) 391–409.
- [11] Y. He, I. Rajantie, M. Ilmonen, T. Mäkinen, M.J. Karkkainen, P. Haiko, P. Salven, K. Alitalo, Preexisting lymphatic endothelium but not endothelial progenitor cells are essential for tumor lymphangiogenesis and lymphatic metastasis, *Cancer Res.* 64 (2004) 3737–3740.
- [12] A. Kadambi, C. Mouta Carreira, C.O. Yun, T.P. Padera, D.E. Dolmans, P. Carmeliet, D. Fukurama, R.K. Jain, Vascular endothelial growth factor (VEGF)-C differentially affects tumor vascular function and leukocyte recruitment: role of VEGF-receptor 2 and host VEGF-A, *Cancer Res.* 61 (2001) 2404–2408.
- [13] T. Hoshida, N. Isaka, J. Hagendoorn, E. di Tomaso, Y.L. Chen, B. Pytowski, D. Fukumura, T.P. Padera, R.K. Jain, Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications, *Cancer Res.* 66 (2006) 8065–8075.
- [14] K. Hayashi, P. Jiang, K. Yamauchi, N. Yamamoto, H. Tsuchiya, K. Tomita, A.R. Moossa, M. Bouvet, R.M. Hoffman, Real-time imaging of tumor-cell shedding and trafficking in lymphatic channels, *Cancer Res.* 67 (2007) 8223–8228.
- [15] M. McElroy, K. Hayashi, B. Garmy-Susini, S. Kaushal, J.A. Varner, A.R. Moossa, R.M. Hoffman, M. Bouvet, Fluorescent LYVE-1 antibody to image dynamically lymphatic trafficking of cancer cells *in vivo*, *J. Surg. Res.* 151 (2009) 68–73.