APPLICATION NOTE

Pre-clinical Imaging

Authors

Jeffrey D. Peterson, Ph.D.

Kristine O. Vasquez

PerkinElmer, Inc.
Waltham, MA USA

Quantitative Pre-clinical Fluorescence Imaging of Cancer Metastasis to the Lung and Response to Therapy

Abstract

Quantitative pre-clinical fluorescence imaging transcends the boundaries of traditional optical imaging of biological structures and physiology by providing information at the molecular level about disease states and therapeutic response. PerkinElmer's proprietary Fluorescent Pre-clinical Imaging Agents and FMT® (Fluorescence Molecular Tomography) Quantitative Pre-clinical Imaging System represent powerful tools for research and drug development in the imaging of biological processes and pharmaceutical activity in living animals. Traditional mouse models of cancer rely primarily on ex vivo measurements of disease morphology and histologic analysis for the assessment of tumor burden. These measurements of disease may be distant from the actual biological targets of interest and can be time consuming, expensive, and impractical for measuring local disease biology in context. Further, these metrics do not enable measurements in living animals and merely focus on changes in shape or size within the affected tissue. In contrast, by using PerkinElmer's near infrared (NIR) imaging agents in combination with the FMT system, the biological processes that change with disease progression and therapeutic response, rather than alterations in morphology, can be visualized non-invasively over time. In this set of studies, ProSense® 750 Fluorescent Pre-clinical Imaging Agent, a protease-activatable NIR probe, effectively imaged

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and quantitated the abnormal increase in protease activity associated with tumor aggression and growth within the lungs of BALB/c mice receiving an intravenous injection of 4T1 mouse breast adenocarcinoma cells. The responses to different chemotherapy treatments were quantitatively measured as a reduction in protease activity that correlated with a decreased tumor burden as assessed by histology. These results indicate that tomographic fluorescence imaging datasets can be established in pre-clinical drug development programs as standards for measuring disease progression and therapeutic response.

Materials and Methods

4T1 Cancer Metastasis Model

Four to five week-old female BALB/c mice were purchased from Charles River Laboratories® (Wilmington, MA) and maintained in a pathogen-free animal facility with water and low-fluorescence mouse chow (Harlan Tekland®, Madison, WI). Handling of mice and experimental procedures were in accordance with PerkinElmer IACUC guidelines and veterinarian requirements for animal care and use. To induce cancer metastasis, BALB/c mice were IV injected with $5x10^5$ 4T1 mouse breast adenocarcinoma cells/100 µL (ATCC®, Manassas, VA). These conditions were optimized to generate measurable lung metastases in the majority of mice within 2 weeks as assessed by tumor burden (determined by lung weight) and fluorescent imaging (described below). The presence of metastatic nodules within the lung parenchyma was confirmed by histology after the lungs were fixed in buffered formalin, sectioned and stained with Hematoxylin and Eosin.

Chemotherapeutic regimen

5-Fluorouracil (5-FU Sigma-Aldrich®, St Louis, MO) is a fluorinated pyrimidine that is metabolized, *in vivo,* to yield 5-fluoro-deoxyuridine monophosphate and other metabolites that block DNA synthesis and cause the production of faulty RNA. 5-FU was resuspended in deionized sterile water and injected intraperitoneally (i.p.) 1x/day starting on the day of tumor cell injection. Mice received 5-FU in combination with 2'-deoxyinosine (dIno, Sigma-Aldrich®), a deoxyribose 1-phosphate precursor with the ability to potentiate 5-FU cytotoxicity and enhance apoptosis. Mice were injected 2x/day i.p. with 1.6 g/kg dIno and 4.4 – 17.5 mg/kg/day 5-FU, for 5 consecutive days. Mice were injected with ProSense 750 agent on day 11 and imaged 24 hrs later on the FMT system as described above.

Imaging agents

At different timepoints after implantation of mice with 4T1 cells, mice were injected with ProSense 750 agent (to detect tumor-related cathepsin activity), IntegriSense™ 680 agent (to detect $\alpha_v\beta_3$ integrin expression), or AngioSense® 680 agent (to detect vascular leak) and imaged by the FMT 2500 LX system 24 hours later.

Lung Imaging Protocol

On the day of imaging, mice were anesthetized, depilated to minimize interference with the fluorescent signal, and positioned in the FMT system imaging chamber. The FMT system's low-power laser diode light source trans-illuminated the tomographic scan region of interest with fluorescent signal captured via a cooled CCD camera fitted with the appropriate optical filters. The collected fluorescence data was reconstructed by TrueQuant™ software for the quantitation of 3-dimensional fluorescence signal within the lung region. The mean fluorescence (in nM) and total amount of fluorescence (in pmoles) were generated for all studies.

Ex vivo imaging

At the termination of each study (day 14) mice were sacrificed and lungs collected, weighed, and assessed macroscopically. Lungs were also imaged on a fluorescence planar imaging system to corroborate the non-invasive in vivo finding. Lungs were subsequently embedded in OCT, frozen, and cut into 7 mm sections which were immediately mounted in DAPI- containing medium for assessment of tissue fluorescence localization.

Statistical analysis

Data are presented as the means ± the standard error of the mean (SEM). Significance analysis of in vivo lung fluorescence was conducted using a two-tailed unpaired Student t test when 2 groups were analyzed or nonparametric ANOVA when 3 or more groups were compared (StatView®, SAS Institute, Cary, NC). Probability values of < 0.05 were considered significant.

Introduction

Metastatic breast cancer remains a clinical challenge today with 210,000 new breast cancer cases per year reported in 2010 in the United States alone (American Cancer Society®). In 10% of breast cancer diagnoses, the cancer has already metastasized to distant organs in the body, such as bone, liver, and lung, decreasing the 5-year relative survival rate to 20%. Thus, it is essential to develop robust in vivo models that can help dissect the metastatic process and assist in the effective development of targeted therapeutic agents.

Over the last 100 years there have been numerous efforts to develop in vivo breast cancer models. These efforts have focused on the establishment of solid tumor models as well as metastatic models that closely mimic the clinical presentation of breast cancer. The establishment of these models, in addition to the development of accurate imaging technologies, have helped in expanding our understanding of tumorigenesis, tumor-host interactions, tumor progression and metastasis. New technologies are also now routinely applied to the elucidation of pharmacologic mechanism of action of new drug entities.

In the present study, we used a well established 4T1 mouse mammary carcinoma model that yields highly invasive metastases to the lungs of normal BALB/c mice upon IV injection. Using the FMT 2500LX system, in conjunction with three different NIR fluorescent probes; we longitudinally tracked and characterized the pulmonary metastatic spread of breast cancer to the lungs as well as quantitated the anti-tumor effect of the chemotherapeutic agent 5-fluorouracil (5-FU). Our non-invasive imaging results were robust and correlated well with terminal assessments such as changes in gross lung weight, ex vivo tissue imaging, and histologic assessment. Such an imaging approach should help to visualize and quantitate steps in cancer progression or metastatic cascade *in vivo,* and will be beneficial in developing novel therapeutic treatments.

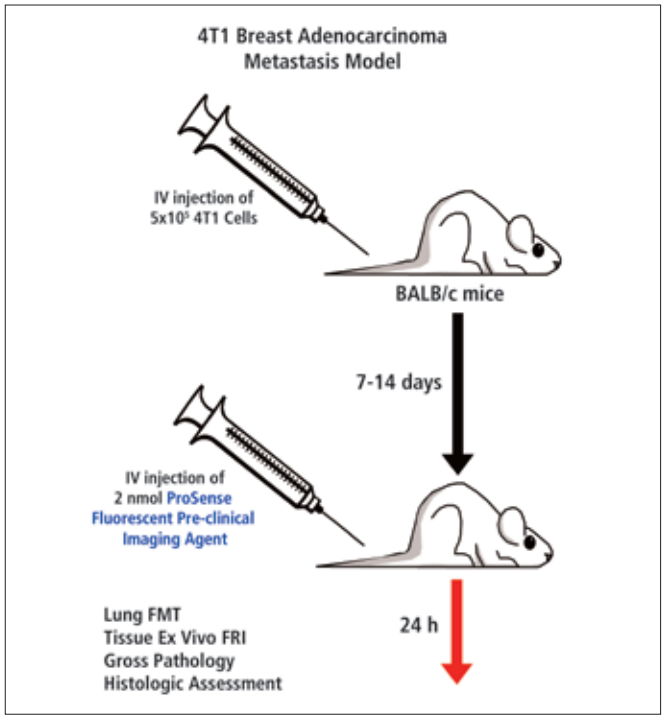


Figure 1. 4T1 lung metastasis model.

Results

4T1 metastasis model

Figure 1 depicts the schematic of the model used in this study, a protocol optimized to generate high incidence of lung metastasis by 14 days. Figure 2 represents the results of a study in which BALB/c mice were injected intravenously with 5x10^5 mouse mammary 4T1 cells. Two weeks later, mice were injected intravenously with the ProSense 750 agent and imaged 24 hours later with the FMT system to generate non-invasive, quantitative imaging. At the end of the imaging session, mice were sacrificed and their lungs observed for macroscopic evidence of abnormalities. In addition, lung tissues were imaged in 2D to validate the levels of ProSense agent fluorescent signal in tissue. Lung metastases were also confirmed by histology after fixation in formalin, sectioning and Hematoxylin and Eosin staining. In situ localization of ProSense agent fluorescence in tissue was performed on frozen sections.

Imaging results show that disease is readily detected in mice receiving injections of 4T1 cells, but only when using the FMT 2500 LX system's tomographic capability and not by whole body 2D imaging. Control mice showed little or no signal other background fluorescence associated with agent metabolism (liver, kidney – not shown). Ex vivo assessment of tissues agreed well with in vivo imaging and quantitation.

Monitoring kinetics of lung metastasis by FMT

Mice were injected/imaged with ProSense agent at four different times following 4T1 cell injection to monitor disease progression. Un-injected mice served as controls (time 0). All imaging was performed 24 hours following intravenous agent injection on the FMT system. No signal could be detected in the lung region of non tumor-injected mice. However, as soon as 1 week following tumor cell injection, discrete areas of near infrared fluorescent ProSense agent signal could be identified in the lung, increasing over time. Quantitation of ProSense agent signal showed statistically higher mean pmols and fluorescent volume (mm^3) in 4T1-bearing mice as compared to un-injected mice (*$p < 0.01$; **$p < 0.005$), with earliest detection of differences seen at 7 days. Total fluorescent signal increased over the next week, reaching peak values on day 14, but the volume of fluorescence per lung did not change much. Results generally agreed with lung weight assessment (which assesses a composite of tissue weight, pulmonary edema, and tumor weight), however, as expected, imaging better reflected the kinetics of tumor growth in vivo.

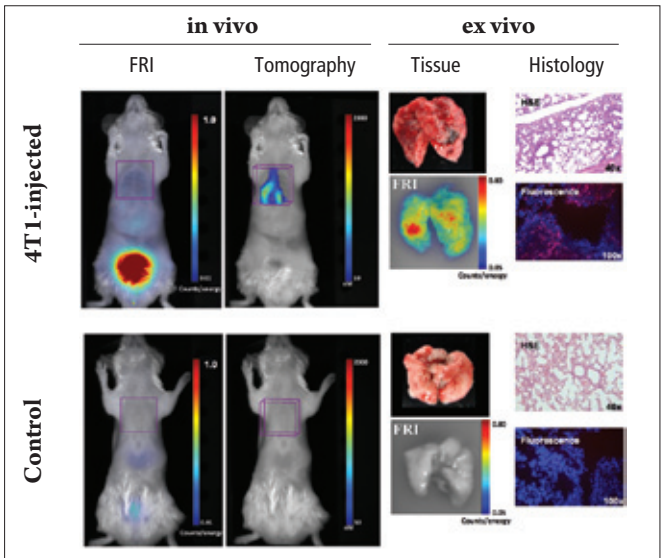
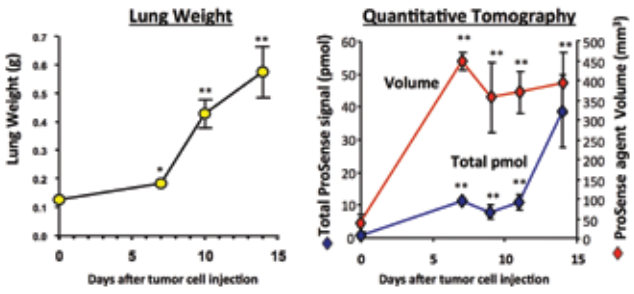


Figure 2. FMT 2500 LX system imaging of tumor cathepsin activity.

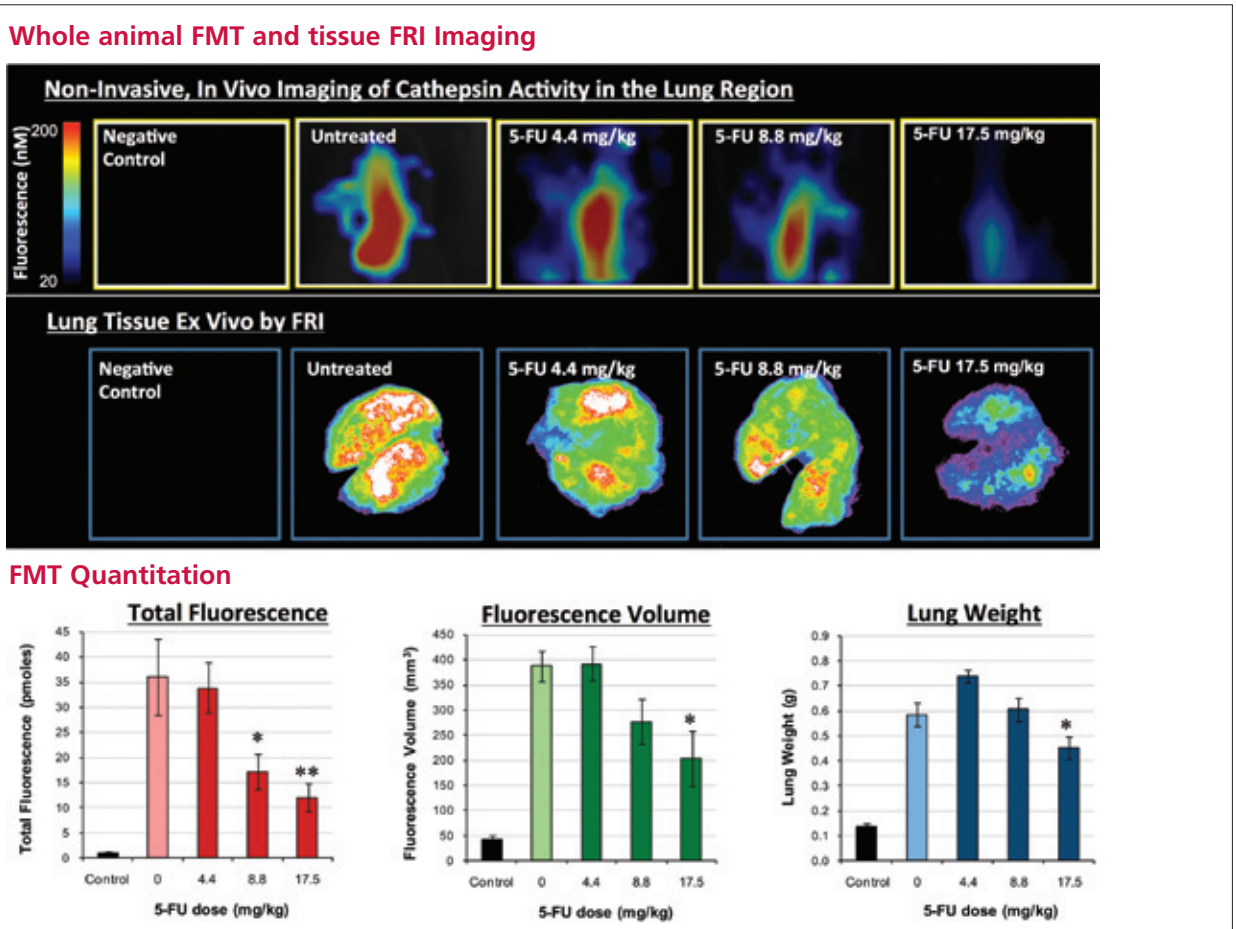


At different times after inoculation with 4T1 cell, mice were injected with ProSense 750 agent and imaged by the FMT system 24 hours later. Analysis of tomographic lung datasets was performed using thresholds based on negative control animals. ProSense agent fluorescence volume and total picomoles within the lungs of 4T1 recipients were quantified by FMT system software.

Figure 3. Quantitation of tumor cathepsin activity.

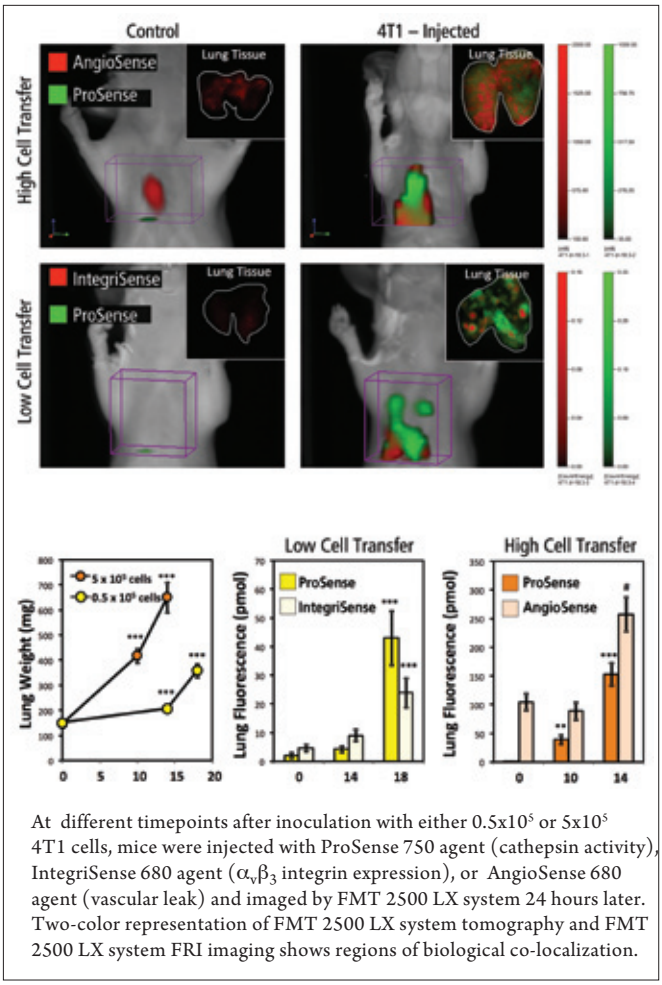
Assessing the 5-FU dose response using the FMT system

To monitor the effect of 5-FU on tumor burden by in vivo fluorescence imaging, mice were IV injected with $5x10^5$ 4T1 cells and dosed with increasing concentrations of 5-FU (4.375, 8.75, and 17.5 mg/kg/day, n = 5 mice per group) on the same day. Mice received 5-FU for an additional 4 days. On day 10, mice were IV injected with ProSense 750 agent and imaged 24 hours later on the FMT system. As shown in Figure 4, tomographic fluorescent signal in the lung region decreased with increasing doses of 5-FU. Figure 5 (left panel) shows a significant decrease of 46% and 63% with doses of 8.75 and 17.5 mg 5-FU/kg/day ($p = 0.02$ and $p = 0.003$, respectively as compared to no treatment; $p = 0.04$ between 4.375 and 8.75 mg/kg/day and $p = 0.006$ between 8.75 and 17.5 mg/kg/day). Fluorescence volume (center panel) provides quantitative data regarding the volume of lung affected by disease, with high dose treatment decreasing this by as approximately 50%. Immediately following the imaging session, mice were sacrificed and their lungs dissected and weighed. With progressively increasing tumor burden, as assessed by lung weight (right panel), a fair correlation between in vivo fluorescence and lung weight was observed (r^2=0.71), however total fluorescence showed much greater sensitivity as a readout of drug efficacy.



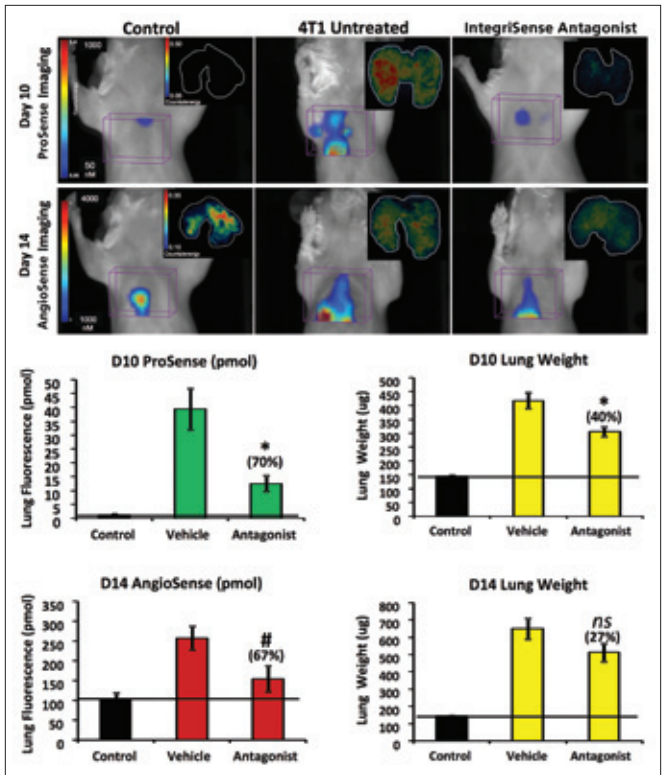
Mice received 5-Fluorouracil (5-FU) + Deoxyinosine (dIno), at doses and dosing regimens indicated, beginning one day following 4T1 cell injection. On day 13, mice received a dose of ProSense 750 agent and were imaged by the FMT system 24 hours later.

Figure 4. Quantitation of 5-FU efficacy with ProSense 750 agent.



At different timepoints after inoculation with either 0.5x10^5 or 5x10^5 4T1 cells, mice were injected with ProSense 750 agent (cathepsin activity), IntegriSense 680 agent ($\alpha_v\beta_3$ integrin expression), or AngioSense 680 agent (vascular leak) and imaged by FMT 2500 LX system 24 hours later. Two-color representation of FMT 2500 LX system tomography and FMT 2500 LX system FRI imaging shows regions of biological co-localization.

Figure 5. Multiplex FMT 2500 LX system quantitation of vascular leak, cathepsin activity, and integrin expression.



Mice received an i.p injection of a small molecule integrin antagonist (25 mpk/mouse/b.i.d.) starting at day 0, starting 1 hour prior to 4T1 cell injection. On days 9 and 13, mice received a combined dose of AngioSense 680 agent and ProSense 750 agent and were imaged by the FMT system 24 hours later.

Figure 6. Multiplex FMT 2500 LX system quantitation of integrin agonist expression.

Multiplex Fluorescence Imaging

To assess the ability of the FMT 2500 LX system to provide dual imaging with simultaneous injection of two imaging agents, both in vivo and in post-mortem tissues (2D), mice were IV injected with 4T1 cells [either 0.5x10^5 ("low 4T1 cell transfer") or 5x10^5 ("high 4T1 cell transfer")] and imaged with combinations of ProSense agent/AngioSense agent or ProSense agent/IntegriSense agent, respectively (Figure 5). The high cell transfer study (upper panels) recapitulated results from Figures 2 and 4, with robust ProSense agent imaging results achieved 14 days after cell transfer. Dual color representation of ProSense agent (tumor and inflammatory cells) and AngioSense agent (vascular leak) revealed wide-spread distribution of both agents throughout the lungs with considerable overlap in the localization of these agents as expected. Using a low 4T1 cell dose (lower panels) and a combination of ProSense agent and IntegriSense agent (tumor, vascular, and inflammatory cells), optimal imaging signal was delayed to day 18 and there were more regions of distinct localization of the two agents. Ex vivo 2D imaging further showed a more nodular distribution of signal, albeit still well distributed widely throughout the lungs.

Quantitation of tumor fluorescence in the lung for these two studies (Figure 6) also incorporated earlier imaging times, showing a clear progression in signal for both high dose and low dose studies. Imaging results for ProSense agent and IntegriSense agent correlated well with lung weights, but the AngioSense agent showed a high background in normal tissue and delayed detection of tumor-induced changes.

Conclusions

Mouse models of cancer metastasis rely predominantly on ex vivo tissue weight, nodule counts, and/or histologic analysis for the assessment of tumor burden. To assess the feasibility of non-invasive, quantitative imaging of deep tissue metastases, we used different NIR imaging agents to image vascular leak, upregulation of cathepsins and increases in integrin expression, known to occur in cancer progression and metastasis. We found that 4T1 cell injection led to diffuse, wide-spread metastases within the lungs of recipient mice, rather than distinct small metastases. Severity of the disease progression was modified using different 4T1 cell doses. Tomographic imaging allowed the quantitation of fluorescent agents accumulating in the tumors, with this quantitation

yielding better results than typical lung weight assessment. Treatment efficacy with either 5-FU or a small molecule integrin antagonist led to robust and quantitative changes, with the additional capacity of measuring multiple biological changes simultaneously. These findings were confirmed by 2D imaging of ex vivo tissue and by histology. With this model we have established the potential utility of quantitative fluorescence tomography in preclinical drug discovery in the challenging area of deep tissue metastasis.

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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