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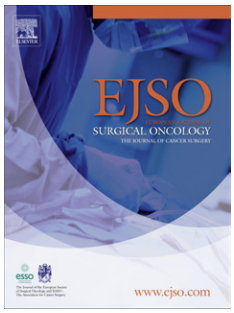

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Intraoperative near-infrared fluorescence imaging of colorectal metastases targeting integrin $\alpha_v\beta_3$ expression in a syngeneic rat model

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Abstract

Aim: Near-infrared (NIR) fluorescence optical imaging is a promising technique to assess the extent of colorectal metastases during curative-intended surgery. However, NIR fluorescence imaging of liver metastases is highly challenging due to hepatic uptake and clearance of many fluorescent dyes. In the current study, the biodistribution and the ability to demarcate liver and peritoneal metastases were assessed during surgery in a syngeneic rat model of colorectal cancer using an integrin $\alpha_v\beta_3$-directed NIR fluorescence probe.

Methods: Liver tumors and peritoneal metastases were induced in 7 male WAG/Rij rats by subcapsular inoculation of 0.5 x10^6 CC531 colorectal cancer rat cells into three distinct liver lobes. Intraoperative and ex vivo fluorescence measurements were performed 24 (N = 3 rats, 7 tumors) and 48 h (N = 4 rats, 9 tumors) after intravenous administration of the integrin $\alpha_v\beta_3$-directed NIR fluorescence probe.

Results: Colorectal metastases had a minimal two-fold higher NIR fluorescence signal than healthy liver tissue and other abdominal organs ($p < 0.001$). The tumor-to-background ratio was independent of time of imaging (24 h vs. 48 h post-injection; $p = 0.31$), which facilitates flexible operation planning in future clinical applications. Total fluorescence intensity was significantly correlated with the size of metastases ($R^2 = 0.92$ for the 24 h group, $R^2 = 0.96$ for the 48 h group).

Conclusion: These results demonstrate that colorectal intra-abdominal metastases can be clearly demarcated during surgery using an integrin $\alpha_v\beta_3$ targeting NIR fluorescence probe. Translating these findings to the clinic will have an excellent potential to substantially improve the quality of cancer surgery.

Keywords: near-infrared fluorescence imaging, image-guided surgery, colorectal cancer, integrin $\alpha_v\beta_3$

Abbreviation used: NIR, near-infrared

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Introduction

Survival of colorectal cancer patients is largely restricted by the occurrence of metastases, predominantly in the liver. In the course of the disease, up to 50% of patients will eventually develop liver metastases.[1] If confined to the liver, surgery offers a possible curative treatment option with 5-year survival rates of 35-40%.[2] However, at the time of surgery, adequate assessment of the extent of the disease is still limited, resulting in a 40-50% recurrence rate.[3] Clearly, innovative visualization techniques are needed to facilitate intraoperative assessment of the extent of the cancer tissue and to guide the subsequent surgical removal of these tumors with adequate margins in an attempt to increase the complete resection rate.

Real-time visualization of cancer cells using near-infrared (NIR) fluorescence optical imaging is a promising technique to assess the extent of colorectal metastases during curative-intended surgery.[4-6] However, optical imaging of liver metastases is highly challenging due to the high absorptivity of liver tissue for visble light and hepatic uptake and clearance of many fluorescent dyes.[7;8] Recent studies demonstrated successful identification of breast, lung and glioblastoma cancers by targeting a member of the integrin family, integrin $\alpha_v\beta_3$.[9-13] Integrins are cell-surface transmembrane heterodimeric glycoproteins that are involved in cell adhesion, matrix interaction and cell signaling pathways. Integrin $\alpha_v\beta_3$ plays a key role in the early phase of tumor angiogenesis, tumor cell migration and is overexpressed in various cancer types, including colorectal cancer.[14-17] Because hepatocytes show little expression of integrin $\alpha_v\beta_3$,[16;18] it is expected that NIR fluorescence probes targeting integrin $\alpha_v\beta_3$ result in little background in the liver.[9] Therefore, the aim of this study was to investigate the biodistribution and the ability to

clearly demarcate liver and peritoneal metastases during surgery in a syngeneic rat model of colorectal cancer using an integrin $\alpha_v\beta_3$-directed NIR fluorescence probe.

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Material and methods

Animal model

Rat CC531 colorectal cancer cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine (Gibco, Invitrogen Ltd, Carlsbad, USA), 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulphate.[19] In order to induce liver and peritoneal metastases, CC531-syngeneic male WAG/Rij rats (Charles River, Maastricht, the Netherlands) weighing 300-350 g underwent median laparotomy and the liver was exposed. Subsequently, 500,000 CC531 cells (50 µl) were subcapsularly inoculated into the left and right main liver lobes, and the right accessory liver lobe.[19] To prevent tumor spill, puncture sites were covered with a small sponge, directly after tumor cell injection. Four weeks after inoculation, metastases of approximately 5 mm in diameter had originated in the liver. The Animal Welfare Committee of the Leiden University Medical Center approved the experiments.

NIR fluorescence probe

The NIR fluorescence probe IntegriSense®680 (VisEn Medical, Woburn, USA) with peak absorbance at 680 nm was used for fluorescence imaging. IntegriSense680 consists of a small non-peptide integrin $\alpha_v\beta_3$ antagonist,[20] which is conjugated to the NIR fluorescence probe VivoTag®-S680 (VisEn Medical).

Intraoperative NIR fluorescence camera system

The Fluobeam® system (Fluoptics, Grenoble, France) was used for these experiments and has been described previously.[21] In short, the Fluobeam system is composed of a 100 mW laser emitting at 690 nm with an illumination power of 2.6 mW/cm^2 and a 12-bit CCD

camera. The animal is placed under the laser and illuminated by white light filtered with a band-pass filter (350-650 nm) providing an irradiance of $7x10^3$ lx at the animal level.

Animal experiments

All animals were housed in the animal facility of the Leiden University Medical Center. Pellet food and fresh tap water were provided ad libitum. The weight of the animals was followed throughout the experiment to monitor their general health state. Throughout tumor inoculation, imaging and surgical procedures, the animals were anesthetized with 5% isoflurane for induction and 2% isoflurane for maintenance in oxygen with a flow of 0.8 L/min and placed on an animal bed with an integrated nose mask. Rats (N = 7) were injected intravenously into the penile vein with IntegriSense680 (12 nmol per animal) 24 h (3 rats, N = 7 tumors) or 48 h (4 rats, N = 9 tumors) before imaging. These time points were chosen based on the blood pharmacokinetics of IntegriSense680 (VisEn website: www.visenmedical.com). Before injection, the level of autofluorescence was determined of metastases, surrounding tissue and abdominal organs. During intraoperative NIR fluorescence imaging of metastases a median laparotomy was performed followed by a systematic exploration of the peritoneal cavity. After intraoperative imaging, livers containing metastases were completely excised for additional *ex vivo* fluorescence measurements. Peritoneal metastases identified clinically or by fluorescence were carefully excised. Fluorescence intensity of metastases and abdominal organs was determined in vivo and ex vivo using the Fluobeam system. After resection, tumors were imaged ex vivo using the IVIS Spectrum (Caliper LifeSciences, Hopkinton, USA), which allowed isolation of the IntegriSense680 signal from the background fluorescence by means of spectral unmixing.[22]

Microscopy

Excised tumors were fixed in 10% buffered formalin overnight and washed in 70% ethanol. Following paraffin embedding and mounting, tissue sections specimens of 4 µm were air-dried and stained with standard hematoxylin-eosin. In parallel, freshly excised liver lobes containing tumors were processed for fluorescence microscopy. Fresh tumors and surrounding liver tissue blocks of approximately 2 cm in diameter were incubated with 0.5 ml phalloidin-Alexa Fluor 488 (0.5 µM) and Hoechst 33342 (48 µM; both from Invitrogen) for 1 hr at room temperature to stain the F-actin filaments in particular those of the cell membrane of hepatocytes,[23] and the dsDNA at the cell nucleus, respectively. Tumors were placed in glass-bottom Petri dish (P35G-1.5-14-C , Mattek Corporation, Ashland, USA) and analyzed using the Leica TCS SP5 inverted confocal microscope (Leica Microsystems, Wetzlar, Germany; HCX PL APO 40x; N.A. 1.25 oil immersion objective). Hoechst, phalloidin-AlexaFluor488 and IntegriSense680 were excited by a 405 nm diode, a 488 nm Argon laser and a 633 HeNe nm laser, respectively. The 12-bit images were analyzed using the Leica LAS AF software and the three signals were pseudo-colored with blue for Hoechst, green for phalloidin-AlexaFluor488, and red for IntegriSense680.

Statistical analysis

Fluobeam derived NIR fluorescence data were analyzed using the open-source software ImageJ[24] by drawing regions of interest at the tumor and at the surrounding tissue within a range of 2 mm of the demarcation line of the tumor and the surrounding tissue by visual interpretation and measuring the fluorescent intensity of the 12-bit images. Merged images of visible light images and NIR fluorescence light images generated by the Fluobeam system were created using Adobe Photoshop CS3 Software (version 10.0.1, Adobe Systems Inc.,

San Jose, USA). IVIS Spectrum derived NIR fluorescence data were analyzed using LivingImage software (version 3.2, Caliper LifeSciences, Hopkinton, USA) using the methods described above. Statistical analysis and generation of graphs were performed using GraphPad Prism Software (version 5.01, La Jolla, USA). Mean fluorescence intensity and associated standard deviations were reported. Unpaired and paired t-tests were used for testing differences of fluorescence intensity between groups. Pearson's correlation coefficients were calculated for the analysis of the size of tumors and the total fluorescence intensity. Statistical tests were two-tailed and $p < 0.05$ was considered significant.

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Results

Intraoperative detection of colorectal liver metastases

In a total of 7 rats, all colorectal liver metastases (N = 16) were identified during surgery with the intraoperative Fluobeam camera system after targeting of integrin $\alpha_v\beta_3$ expression with IntegriSense680 (Fig. 1A). Quantification of the NIR fluorescence signal was performed using spectral unmixing with the IVIS Spectrum, enabling separation of true IntegriSense680 signal from background fluorescence. The NIR fluorescence signal was significantly higher in the colorectal metastases in comparison to healthy liver tissue, regardless of the rats were imaged 24 hours after injection (N = 7, paired t = 10.8, $p < 0.0001$) or 48 hours after injection (N = 9, paired t = 6.59, p = 0.0002; Fig. 1B). The NIR fluorescence signal of the colorectal metastases was on average two-fold higher than that of the healthy liver and the tumor-to-background ratio was not significantly different when comparing the 24 hours (mean TBR = 2.04) and 48 hours group (mean TBR = 1.81, unpaired t = 1.06, p = 0.31; Fig. 1C). Total fluorescence intensity was significantly correlated with the size of metastases (R^2 = 0.92 for the 24 h group, R^2 = 0.96 for the 48 h group). Measurements on the IVIS Spectrum were in concordance with the visual information provided by the Fluobeam system (data not shown). Sectioning of tumor and liver tissue showed a clear demarcation of fluorescence signal at the tumor border, which was confirmed by fluorescence microscopy (Fig. 2).

Biodistribution of IntegriSense680

To assess the potential use of IntegriSense680 in intraoperative identification of colorectal metastases in close proximity to other organs in the peritoneal cavity, the NIR fluorescence intensity of abdominal organs was quantified 24 and 48 hours after injection of IntegriSense680 using the Fluobeam system (Fig. 3). All abdominal organs showed

fluorescence intensity levels comparable to or lower than the liver. No difference was observed between measurements at 24 and 48 hours after injection.

Intraoperative detection of intra-peritoneal metastases

The sufficiently low fluorescence levels of abdominal organs suggest a potential use of IntegriSense680 for identification of peritoneal colorectal metastases. In all animals, the peritoneal cavity was carefully inspected for tumor deposits. In several animals, several suspected tumors were found in the mesentery (Fig. 4). These nodules were fluorescent and were confirmed as malignancies by histological analyses. Total fluorescence was significantly correlated with the size of mesenterial metastases ($R^2 = 0.99$). No clinically evident intra-abdominal tumor deposits without fluorescence were observed.

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Discussion

NIR fluorescence imaging

We have demonstrated the potential use of the NIR fluorescence probe IntegriSense680 for intraoperative tumor identification in both colorectal metastases confined to the liver and metastases elsewhere in the peritoneal cavity. To our knowledge, the current study is the first study on targeting liver metastases using a targeted NIR fluorophore. NIR fluorescence imaging within the peritoneal cavity is often hampered by high levels of background fluorescence. Despite these difficulties, IntegriSense680 provided an adequate tumor-to-background ratio to identify tumors in the vicinity of abdominal organs. The targeting ligand of IntegriSense680 is a non-peptide integrin $\alpha_v\beta_3$ antagonist that is derived from the full-length RGD tripeptide.[20] This non-peptide molecule has a significantly improved affinity in comparison with the full-length RGD peptides, which have been used in imaging studies.[10;25-27] No difference was observed between imaging 24 hours post administration and imaging 48 hours post administration. This broad window of intraoperative imaging facilitates flexible operation planning in future clinical applications.

Peritoneal metastases

In the current study, we were able to detect both liver and peritoneal metastases. Although the model is set up as a liver tumor model, some rats also developed peritoneal metastases, particularly those bearing larger liver tumors. Peritoneal spreading of tumor cells could have derived from tumor spill during the inoculation procedure or "real" metastases from large liver tumors.[28] Although, this may be a biologically relevant difference, it possesses no alteration for the purpose of the current study. During the experiments, no clinically evident intra-abdominal or lung metastases were found that were not NIR fluorescent.

It is well known that small metastases can easily be missed on preoperative imaging studies, as CT or MRI. Previously, a minimal detectable number of tumor cells of approximately 13,000 was reported with the CC531 colorectal cancer cell line and NIR fluorescence imaging,[21] reflecting submillimeter tumor depositions. NIR fluorescence imaging could therefore potentially be used in the clinic as an adjunct to preoperative staging, for intraoperative detection of metastases that were not observed on preoperative tumor dissemination imaging.

Clinical implications

Integrin $\alpha_v\beta_3$ as a targeting ligand has been studied extensively in a preclinical setting.[18;29;30] Several clinical studies have been performed using integrin $\alpha_v\beta_3$ as a target for radioimmunotherapy in breast cancer and glioblastoma patients.[11;25] Although radiotracers are potentially useful for diagnosis and treatment by means of radioimmunotherapy, their applicability during surgery is limited due to a lack of intraoperative visualization of the tracer. Nonetheless, these studies suggest a successful introduction of integrin $\alpha_v\beta_3$ as a targeting ligand for NIR fluorescence image-guided surgery in the clinic, which will enable real-time, intraoperative visualization of tumor cells. A major hurdle to be taken is clinical approval of novel NIR fluorophores. Currently, indocyanine green and methylene blue are the sole NIR fluorophores available for clinical applications. Both compounds have suboptimal properties for intraoperative applications and both are not easily conjugated to a targeting ligand. Ishizawa and colleagues have demonstrated the possibility of intraoperative identification of colorectal liver metastases after intravenous injection of indocyanine green.[31] In this study, colorectal liver metastases displayed a fluorescence pattern described as “rim fluorescence”, where the tumor itself is not fluorescent, but the surrounding liver tissue is. Ishizawa et al suggest that this is caused by

decreased biliary excretion of indocyanine green around the tumor. Whereas this is an interesting observation for the detection of hepatic colorectal metastases, the mechanism exploited for this purpose (biliary excretion) prevents this from being used for extra-hepatic intra-abdominal colorectal metastases. This is of particular importance, as the detection of extra-hepatic metastases is a determining factor for clinical decision making. Furthermore, the efficacy of this probe for tumor imaging is strongly dependent on biliary excretion and liver function, limiting its applicability in some patients. Many research groups are working on advancing novel NIR fluorescence fluorophores to the clinic. When these agents become available for clinical testing, NIR fluorescence image-guided surgery will have the potential to greatly improve surgical practice and patient outcome.

Conclusion

NIR fluorescence image-guided surgery has the potential to improve surgical oncology by addressing one of its most fundamental challenges, the complete and en bloc resection of tumors. The current study demonstrates the feasibility of employing a novel NIR fluorescence contrast agent, IntegriSense680, for the intraoperative detection of colorectal metastases in a syngeneic rat model. Clinical translation of NIR fluorescence agents such as IntegriSense680 is pivotal for improving the quality of cancer surgery.

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Conflict of interest statement

None to declare.

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Figure Legends

Figure 1 - NIR fluorescence detection of colorectal liver metastases using IntegriSense680:

A. Intraoperative NIR fluorescence detection of a 1.2 * 5 mm CC531 colorectal liver metastasis in a male rat 24 h after injection of 12 nmol IntegriSense680. Camera exposure time was 15 ms.
B. The NIR fluorescence signal of colorectal liver metastases and healthy liver tissue is plotted for rats injected with IntegriSense680 after 24 h (N = 3 rats, 7 tumors) and 48 h (N = 4 rats, 9 tumors). P values represent paired t-test comparisons.
C. Tumor-to-background ratio is plotted for rats injected with IntegriSense680 after 24 h and 48 h. There was no significant difference between the groups (unpaired t-test, t = 1.06, p = 0.31).

Figure 2 - *Ex vivo* NIR fluorescence imaging of a colorectal liver metastasis:

Shown are a color image (left), a NIR fluorescence image (middle left), and a pseudocolored green merge (middle right) of a 0.9 * 1.8 cm CC531 colorectal liver metastasis within an excised liver lobe. Imaging was performed 48 h after injection of 12 nmol IntegriSense680. Camera exposure time was 10 ms. The distance between two major ruler ticks is 1 cm. *Ex vivo* fluorescence microscopy (right) of a freshly resected liver lobe containing a CC531 colorectal liver metastasis after incubation with Hoechst (dsDNA, pseudo-colored blue) and phalloidin-AlexaFluor488 (F-actin filaments, pseudo-colored green). The IntegriSense680 signal is pseudo-colored red. The dashed line indicates the transition zone between the normal liver (left bottom corner) and the liver tumor (right upper corner).

Figure 3 - In vivo biodistribution of IntegriSense680:

Fluorescence intensity of colorectal CC531 liver tumors and abdominal organs was measured using the Fluobeam camera system in two rats before (level of autofluorescence), 24 h and 48 h after administration of 12 nmol IntegriSense680. Camera exposure time was 10 ms. Bars represent mean ± SD.

Figure 4 - NIR fluorescence imaging of colorectal CC531 mesenterial metastases using IntegriSense680 and the intraoperative camera system:

Shown are a color image (top left), a NIR fluorescence image (top middle), and a pseudocolored green merge of the two images (top right) of a part of the jejunum and accompanied mesentery of a rat bearing multiple CC531 mesenterial metastases. A – E indicate suspected metastases, LN indicates mesenteric lymph nodes, Neg indicates area selected as negative control). Imaging was performed 24 h after injection of 12 nmol IntegriSense680. Camera exposure time was 20 ms. The distance between two major ruler ticks is 1 cm. H&E staining (bottom left) of a 4 µm formalin-fixed, paraffin-embedded tissue section of the metastasis indicated by B (dashed square, top right). The size, NIR fluorescence measurements and presence of tumor cells of the indicated regions (top right) are tabulated at the bottom right panel. Total NIRF is the total NIR fluorescence signal of the region-of-interest. Average NIRF is the total NIR fluorescence signal of the region-of-interest divided by the area. Total NIR fluorescence was significantly correlated with the size of the metastases ($R^2 = 0.99$).

Figure 1
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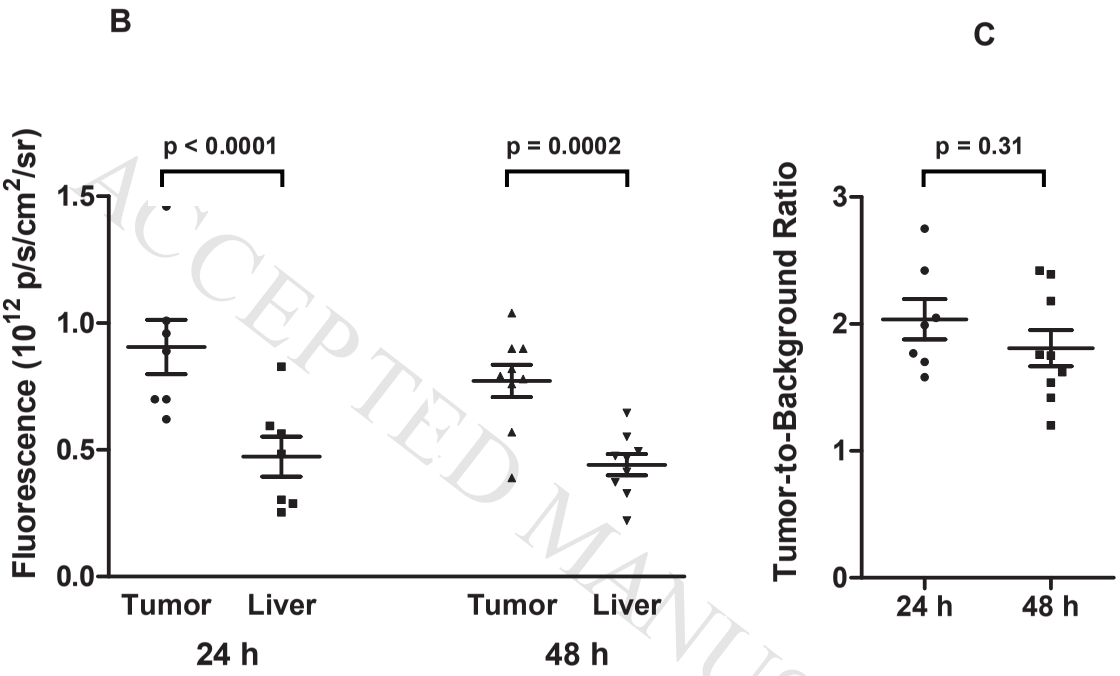

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Figure 2
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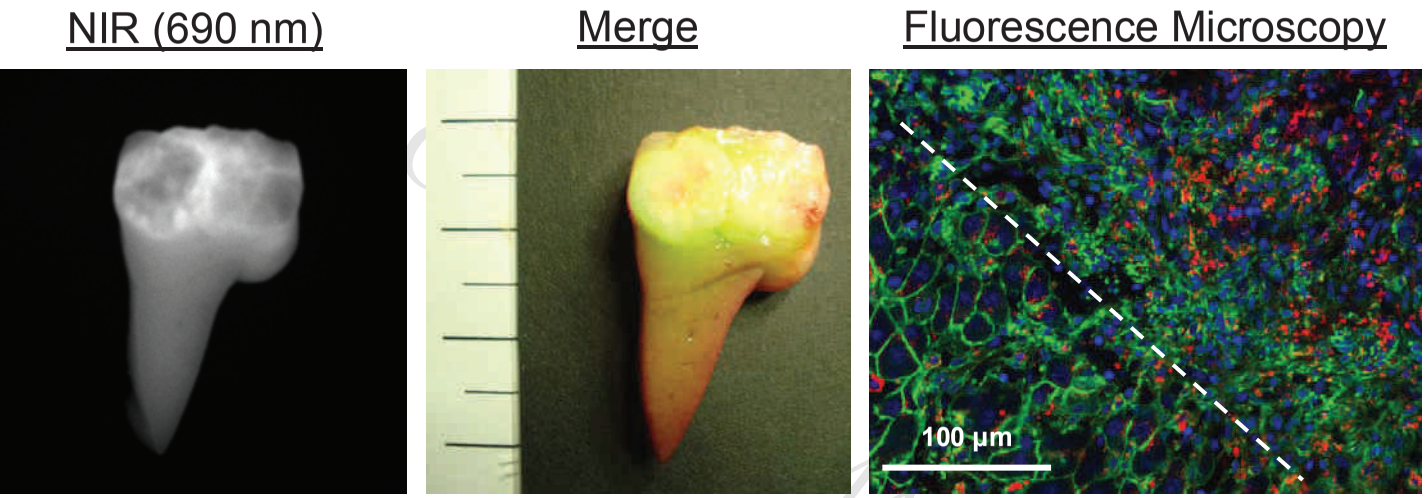

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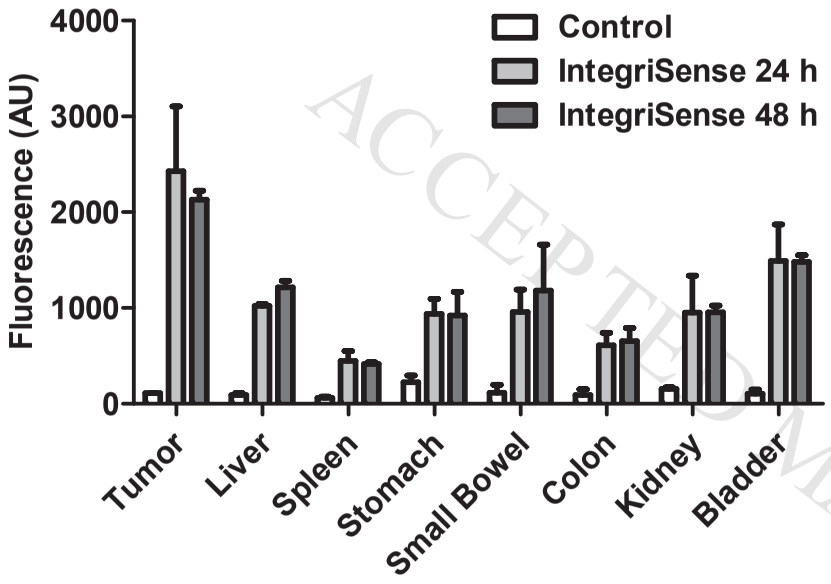
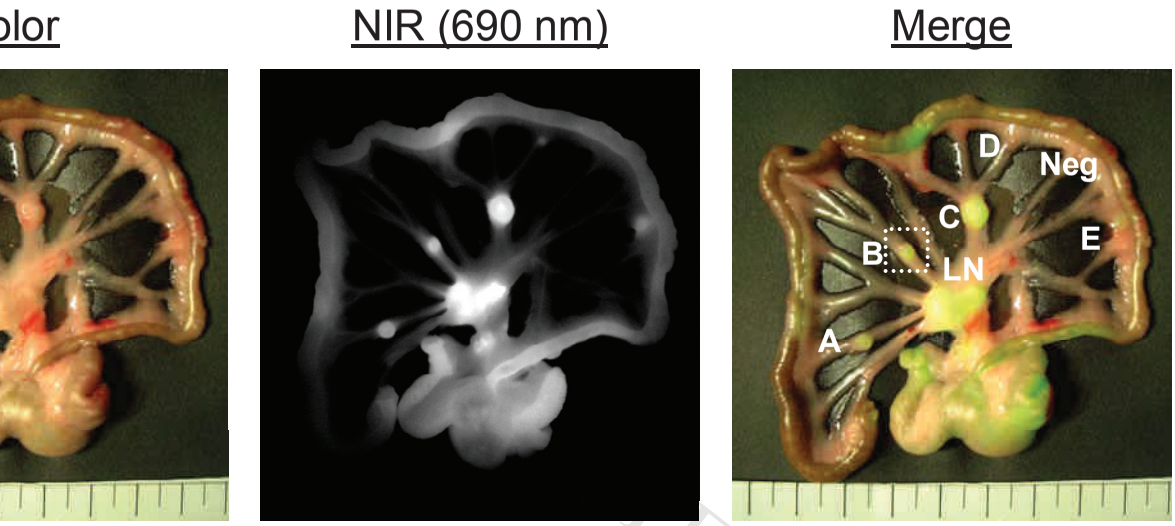
Figure 3
Mieog and Hutteman et al.

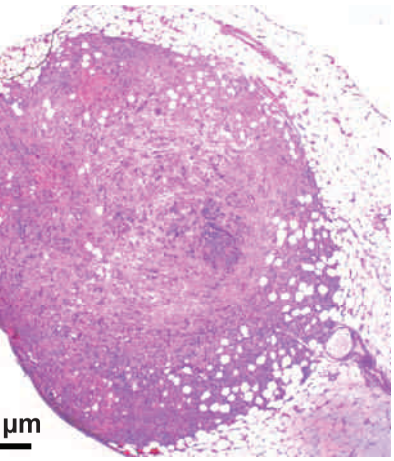


ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure 4
Mieog and Hutteman et al.





	Size (mm)	Total NIRF	Average NIRF	Tumor
A	3.4 x 2.5	199.4	779.0	+
B	2.3 x 2.0	163.1	1101.7	+
C	5.0 x 4.8	1024.8	1362.8	+
D	0.8 x 0.8	24.2	302.5	+
E	1.7 x 1.3	137.2	581.4	+
LN	10.6 x 6.0	3504.4	1633.8	+
Neg	0.9 x 0.9	10.6	173.7	-

ACCEPTED MANUSCRIPT