Published in final edited form as:
Ann Surg Oncol. 2010 April ; 17(4): 1094–1100. doi:10.1245/s10434-009-0868-8.

Intraoperative Localization of Insulinoma and Normal Pancreas using Invisible Near-Infrared Fluorescent Light

Joshua Winer, M.D.[1], **Hak Soo Choi, Ph.D.**[2], **Summer L. Gibbs-Strauss, Ph.D.**[2], **Yoshitomo Ashitate, M.D.**[2], **Yolonda L. Colson, M.D., Ph.D.**[1], and **John V. Frangioni, M.D., Ph.D.**[2,3,*]

[1] Department of Surgery, Brigham and Women's Hospital, Boston, MA 02115

[2] Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215

[3] Department of Radiology, Beth Israel Deaconess Medical Center, Boston, MA 02215

Abstract

Background: Neuroendocrine tumors of the pancreas, such as insulinoma, are difficult to localize and complete resection is essential for cure. Our hypothesis is that a near-infrared (NIR) fluorophore exhibiting uptake in insulinoma could provide high sensitivity detection intraoperatively.

Methods: The optical properties of methylene blue (MB) were measured *in vitro* in 100% serum at 37°C, and *in vivo* after tissue uptake. MB was injected as a rapid intravenous bolus at doses ranging from 0.25 to 2 mg/kg into wildtype rats and pigs, and into insulinoma-bearing transgenic mice. The FLARE™ imaging system was used to acquire color video and NIR fluorescence images simultaneously, and in real-time. The signal-to-background ratios (SBR) of tissues and tumors were quantified using FLARE™ software.

Results: When appropriately diluted, MB exhibits moderate NIR fluorescence emission peaking at 688 nm. At doses ≥ 1 mg/kg, certain normal tissues, such as pancreas, accumulate MB and remain NIR fluorescent for up to 1 hr with an SBR ≥ 1.6. MB spectral properties are maintained after uptake into tissue. Interestingly, insulinoma exhibits even higher uptake for MB than normal pancreas, resulting in insulinoma-to-pancreas ratios of 3.7 and insulinoma-to-muscle ratios of 16.2. MB permitted high-sensitivity, real-time localization of primary, multi-centric, and metastatic insulinoma, and permitted differentiation among tumor, normal pancreas, and other abdominal structures.

Conclusion: A single intravenous injection of a clinically available, commonly used NIR fluorophore provides prolonged intraoperative localization of normal pancreas and insulinoma using invisible NIR fluorescent light.

Keywords

Intraoperative Imaging; Near-Infrared Fluorescence; Methylene Blue; Pancreas; Neuroendocrine Tumors

INTRODUCTION

Neuroendocrine tumors of the pancreas and duodenum are comprised of gastrinomas, VIPomas, somatostatinomas, and insulinomas, with the latter accounting for 17% of all cases.

*Corresponding Author: John V. Frangioni, M.D., Ph.D., Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Room SL-B05, Boston, MA 02215, Phone: 617-667-0692 Fax: 617-667-0981, jfrangio@bidmc.harvard.edu.

1–3 The majority of pancreatic neuroendocrine tumors (PETs) are functional, as is the case for insulinoma, and/or malignant, and require complete resection for cure.[4]

Because these tumors tend to be small, the clinical challenge lies in their localization. To date, multiple pre-operative and intraoperative tests have been utilized, including computed tomography (CT),5 magnetic resonance imaging (MRI),5 transhepatic venous sampling after intra-arterial calcium stimulation,6 receptor-specific radioscintigraphy,7 and ultrasonography (US).2 Recent studies suggest pre-operative and intraoperative detection rates ≥ 92%.2 However, despite such advances, intraoperative localization of tumors less than 2 cm remains difficult, and few methods described to date provide specific contrast intraoperatively.

One such method for localizing insulinoma intraoperatively requires cannulation of arterial feeding vessels followed by injection of high-dose methylene blue (MB).[8–10] MB is an FDA-approved agent used at millimolar concentrations as a visible blue dye in the staining of parathyroid.11 For unclear reasons, MB also stains insulinoma after arterial injection.12 However, intra-arterial injection is cumbersome, time-consuming, and has the potential for patient morbidity.

Recently, our group has published a study showing that when appropriately diluted, to levels that are almost clear to the human eye, MB becomes a moderate strength fluorophore emitting at ≈ 700 nm in the near-infrared (NIR).13 And, when injected intravenously (IV), certain tissues, such as myocardium, efficiently extract MB from the bloodstream and become brightly NIR fluorescent.13 NIR wavelengths of light, invisible to the human eye, also penetrate relatively deeply into tissue but do not change the look of the surgical field (reviewed in 14). They are thus ideal for “highlighting” specific objects in the surgical field, and for seeing “through” several millimeters of living tissue.

Since neuroendocrine tumor resection, typified by insulinoma, is presently performed without real-time, optical localization of tumors, we hypothesized that the combination of a NIR fluorescent contrast agent and an appropriate imaging system could help the surgeon localize insulinoma, define the boundaries of the pancreas, and find small occult metastases within the surgical field.

MATERIALS AND METHODS

Reagents

Methylene blue injection USP (1%; 10 mg/ml) was from Taylor Pharmaceuticals (Buffalo Grove, IL).

Measurement of *In Vitro* and *In Vivo* Optical Properties

Absorbance and fluorescence were measured using fiberoptic HR2000 absorbance (200 – 1100 nm) and USB2000FL fluorescence (350 – 1000 nm) spectrometers (Ocean Optics, Dunedin, FL). NIR excitation was provided by a 5 mW, 655 nm laser diode. All optical measurements of MB were made in 100% fetal bovine serum (FBS) buffered with 20 mM HEPES at 37°C. For fluorescence quantum yield (QY) measurements, oxazine 725 in ethylene glycol (QY = 19%15) was used as a calibration standard, under conditions of matched absorbance at 655 nm. For *in vivo* measurement of MB absorbance and fluorescence properties, a model R400-7-VIS/NIR 400 μm reflection probe (Ocean Optics) was employed.

Intraoperative NIR Fluorescence Imaging

The FLARE™ intraoperative fluorescence imaging system has been described in detail previously.16 Lighting was from an LED-based light source[17] providing 40,000 lux of white

(400 – 650 nm) light and 2 mW/cm^2 (rats) and 4 mW/cm^2 (pigs) of 670 nm NIR excitation light over a 15-cm diameter field-of-view (FOV). Color video and NIR fluorescence images were acquired simultaneously using custom software,[16] which also provided quantitation of the signal-to-background ratio (SBR) of NIR fluorescence intensity. Imaging was performed pre-injection, then continuously over 60 min, using an NIR fluorescence camera exposure time of 150 to 250 msec.

Animal Model Systems

Animals were studied under the supervision of an approved institutional protocol. Female Yorkshire pigs (n = 4), averaging 35 kg, were from E. M. Parsons and Sons (Hadley, MA). Pigs were induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Labs, Fort Dodge, IA), intubated, and maintained with 2% isoflurane (Baxter Healthcare, Deerfield, IL). Body temperature, pulse oximetry, and electrocardiogram were monitored continuously. A midline laparotomy was performed to expose the viscera. The desired dose of MB was diluted into 5 ml of saline and administered as a rapid (≤ 20 sec) IV bolus.

Male Sprague-Dawley rats (n = 15), averaging 325 g, were from Taconic Farms (Germantown, NY). Rats were anesthetized with 60 mg/kg intraperitoneal pentobarbital, and a midline laparotomy was performed to expose the viscera. The desired dose of MB was diluted in 200 μL of saline and administered as a rapid (≤ 5 sec) IV bolus. For pancreas SBR measurements in rat, an unpaired Student's t-test was employed to determine the statistical difference between two groups with a 95% confidence interval. An analysis of variance was used to analyze three or more groups. Correlation was studied using the two-tailed Pearson test with a 95% confidence interval.

Insulinoma-bearing NOD/ShiLt-Tg(RipTAg)1Lt/J mice[18] (n = 8) were from Jackson Laboratories (Bar Harbor, ME). Mice were received at 6 to 8 weeks of age and were observed on a high glucose (1 sugar cube in 250 ml H_2O = 93 mM) diet until they began to appear lethargic (≈ 10 to 14 weeks of age), suggesting the presence of one or more insulinomas. For imaging, mice were anesthetized with 50 mg/kg intraperitoneal pentobarbital, and a midline laparotomy was performed to expose the viscera. The desired dose of MB was diluted in 100 μL of saline and administered as a rapid (≤ 5 sec) IV bolus. After imaging, tissues were resected, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), and flash frozen in liquid nitrogen. Tissue was cryo-sectioned at 10 μm intervals, and consecutive sections were stained with hematoxylin and eosin (H&E) or left unstained for visualization under a Nikon (Melville, NY) TE300 fluorescence microscope.

RESULTS

In Vitro Optical Properties of MB in 100% Serum at 37°C

The chemical structure of the oxidized, colored form of MB used clinically is shown in Figure 1A. In 100% serum, MB was a moderate strength NIR fluorophore, exhibiting peak absorbance at 665 nm, an extinction coefficient at peak absorbance of 71,200 $M^{-1}cm^{-1}$, peak fluorescence emission at 688 nm, and a QY of 3.8% (Figure 1B).

In Vivo Imaging of Normal Pancreas Using MB

The FLARE™ imaging system[16] was configured (Figure 1C) to acquire color video and 700 nm NIR fluorescence images simultaneously and in real-time. Preliminary experiments in rats (Figure 2A) suggested that a single IV injection of MB, in the range of ≈ 1 mg/kg, resulted in NIR fluorescence of normal pancreas.

To confirm that these results were not species-specific, 35 kg Yorkshire pigs (n = 4) were administered 1.5 mg/kg MB and their viscera imaged pre-injection and for 60 min post-injection. Immediately after injection, most of the abdominal viscera became NIR fluorescent, however, by 5 min, MB had cleared sufficiently from surrounding tissues and organs such that the pancreas was highlighted. From 5 to 15 min (Figure 3A), as MB was cleared, NIR fluorescence increased in the small bowel (from excretion into bile) and kidney (from excretion into urine). The NIR fluorescent signal in the pancreas was maintained at a relatively constant level, with an SBR (pancreas to kidney) of ≈ 3.0, for approximately 60 min post-injection.

The highest SBR was achieved when MB was administered as a rapid bolus (over 5 to 20 sec) rather than a slow bolus (over 15–20 min; data not shown), although both techniques provided contrast to the pancreas.

Optimization of Dose and Optical Properties of MB in Pancreas

To quantify NIR fluorescence signal intensity of the pancreas, and to optimize the dose, n = 3 rats per group were administered MB at doses of 0.25, 0.5. 1.0, or 2.0 mg/kg. Only doses of 1 mg/kg and higher resulted in an SBR ≥ 1 and a statistically significant ($p < 0.05$) difference from baseline (Figure 2B). Although there was a trend towards improved SBR at higher doses, there was no statistical difference between 1 mg/kg and 2 mg/kg, suggesting that a plateau in signal strength had been reached, either due to fluorescence quenching at higher extracted concentration or saturation of the uptake process.

In situ reflectance absorbance and fluorescence spectrometry of the pancreas in pig suggested that MB extracted from the bloodstream was chemically unchanged (Figure 3B).

Intraoperative Localization of Insulinoma

Eight insulinoma-bearing mice were imaged pre-injection, then continuously for 60 min post-injection of 1.5 mg/kg MB given as a rapid IV bolus. Tumors fluoresced immediately upon injection and within 2 min were easily distinguished from surrounding pancreas (Figure 4). High SBR was maintained for approximately 60 min. At 15 min post-injection, the ratio of signal intensity in tumors relative to normal pancreas was 3.7 ± 0.5, and in tumors relative to muscle was 16.2 ± 2.8. Importantly, MB provided the surgeon with the ability to visualize primary tumors, multi-centric tumors, and metastatic tumors, even when less than 1 mm in size (Figure 4). Indeed, many of the metastases found intraoperatively were otherwise occult, including those attached to the diaphragm and epigastrium. The FLARE™ imaging system also provided real-time image guidance during tumor resection, and permitted the surgical field to be inspected with high sensitivity to confirm complete resection (data not shown).

Histological Analysis of MB Uptake into Normal Pancreas and Insulinoma

Histological evaluation of specimens obtained 15 min post-injection confirmed that MB was taken up by cells of the exocrine pancreas and pancreatic islets nearly equally (Figure 5A), and that at the cellular level, insulinoma did not have increased uptake relative to normal pancreas (Figure 5B). These results suggest that the perfusion of insulinoma and normal pancreas do not differ significantly, and that the improved tumor-to-normal ratio seen *in vivo* is a consequence of dense cellular packing and NIR light's ability to "integrate" signal by penetrating several millimeters into tissue.

DISCUSSION

The preoperative and intraoperative localization of neuroendocrine tumors of the duodenum and pancreas consumes time and resources. Even with the latest advances, there remain multiple tumors that are not effectively identified, leading to incomplete or blind resection,

recurrent disease, and/or the need for re-exploration. Computed tomography and MRI have been employed with varying success rates depending on the tumor subtype. These imaging modalities have a very high rate of detection for larger tumors,[3] but the results for smaller tumors are not as good.[1,10,19,20]

The gold standard for preoperative localization is somatostatin receptor radioscintigraphy (SS-R), which has a sensitivity 80 to 90% for all PETs except insulinoma.[3] This test is based on the expression of somatostatin receptor type 2, which is the predominant receptor subtype expressed by some PETs. Insulinomas and other tumors that do not express this receptor may require provocative arteriography/venography for localization. These tests, though, can be difficult to interpret in the setting of anatomic variation or dual blood supply to regions of the pancreas.2,21 Ultrasound and palpation are also used intraoperatively to assist with tumor localization, with some groups reporting excellent results.2

Even with these techniques, failure to localize PETs occurs in up to 30% of patients with gastrinoma and 10% with insulinoma.[10] Because of the difficulty in finding smaller tumors, alternative methods have been developed, including intraoperative staining of tumors with vital dyes. Gordon,[22,23] Keaveny[11,12,24] and colleagues demonstrated that there was high uptake of MB in parathyroid and neuroendocrine tumors after bolus IV administration, with large doses staining both primary tumors and metastases. Prinz and colleagues modernized this method in the 1990s by combining MB injection with super-selective arterial cannulation of the feeding vessel of the tumor.[8–10] They demonstrated that even small PETs could be identified with this technique and that curative resections could be performed. However, since the technique required both pre-operative and intraoperative celiac angiograms, and only stained a small region of the pancreas, it was not widely adopted.

Invisible NIR fluorescent light, capable of penetrating millimeters into living tissue, offers several advantages for image-guided PET surgery including no change to the naked eye appearance of the surgical field, high resolution imaging, and high sensitivity imaging. Enabling this technology are recent advancements in intraoperative imaging systems, including the FLARE™ system,[16] which is capable of displaying surgical anatomy with two independent channels of NIR fluorescence simultaneously. In this study, we utilized only a single channel (700 nm) of NIR fluorescence, thus leaving the second, 800 nm channel for imaging of vasculature, nerves, or any other desired structure.

Unlike arterial cannulation, MB-based NIR fluorescence of normal pancreas and insulinoma required only a simple IV bolus injection, and adequate time (2–5 min) for clearance from other abdominal tissues and organs. Moreover, NIR fluorescence signal intensity was prolonged (up to 60 min) and could be repeated as needed. Intraoperative “highlighting” of normal pancreas might be useful during abdominal procedures where there is risk of damage to the pancreas, such as colonic surgery involving the splenic flexure, or in the setting of adhesions and inflammation, where the boundary of the pancreas, and especially the tail, can be obscured. Interpreting MB NIR fluorescence signal in the abdomen, though, requires an understanding of normal MB elimination routes (i.e., into bile and urine), which over time will highlight small bowel, extrahepatic bile ducts, and ureters, in addition to the pancreas (see, for example, Figure 3A).

The MB dose required for imaging, 1 to 2 mg/kg, is consistent with how the drug is now used clinically. As an intraoperative guide for parathyroid resection,[11,12,22–24] and for treatment of acute methemoglobinemia, MB is typically dosed at 1 to 5 mg/kg, and for treatment of ifosfamide-induced encephalopathy, dosing can be 50 times higher.25 However, the rate of dose administration requires caution. Our data suggest that a rapid bolus (over 15 to 20 sec) is optimal, which is consistent with cellular uptake of a perfusion tracer, and previous studies in

dogs.26 MB is typically given as a slower bolus over 15 to 20 min. Although we did not see circulatory collapse in any of our animal studies, rapid MB injection causes a transient, presumably false-negative, fall in pulse oximetry values (pseudohypoxia26). Clinical translation of the results presented in our study should begin with defining the minimum rate of MB dose administration needed for optimal imaging in humans. MB should also be avoided in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, where drug-induced hemolytic anemia can occur.

Despite our encouraging results, many issues remain unresolved. First, it is unknown whether this technology will work with other pancreatic neuroendocrine tumors, such as gastrinomas, VIPomas, or somatostatinomas, although previous data from Prinz and colleagues suggest that it might.[8–10] Second, our focus on MB as the NIR fluorophore of choice was based solely on practicality. MB is not an ideal fluorophore for imaging either the pancreas or insulinoma. It has only modest optical properties, is extracted by many other tissues and organs including myocardium,[13] is not preferentially extracted from the blood by islet cells (Figure 5A) or insulinoma (Figure 5B), emits at 700 nm rather than the more desirable 800 nm, and may require a bowel cleansing regimen to remove autofluorescent food particles from the gut. However, because it is already FDA-approved for other indications, clinical translation of our results in animals could potentially occur more expeditiously than for a new chemical entity.

Our study is a step forward on the path towards personalized medicine and image-guided cancer surgery. Even with arterial cannulation, patient-to-patient anatomic variation of the blood supply to the pancreas can render the technique ineffective for finding insulinoma.[2,21] Being able to create tumor contrast using IV injection of a contrast agent eliminates this problem, and also provides the oncologic surgeon with unprecedented guidance during localization and resection of small tumors and occult metastases. As shown in Figure 4, even millimeter-sized multi-centric and metastatic tumors were clearly visible, and completeness of resection could be assessed in real-time.

In conclusion, we have developed an optical imaging technique that employs invisible NIR fluorescent light to define the boundaries of normal pancreas, and to localize insulinomas, after a single IV injection of an agent already FDA-approved for other indications. NIR fluorescence signal intensity remains high for up to 60 min, and injections can be repeated, thus providing prolonged, high-sensitivity, and real-time intraoperative guidance.

Acknowledgments

We thank Barbara L. Clough for editing and Lorissa A. Moffitt for administrative assistance. This study was supported by Bioengineering Research Partnership grant #R01-CA-115296 to JVF from the National Institutes of Health (National Cancer Institute).

Financial Disclosure: This study was supported by Bioengineering Research Partnership grant #R01-CA-115296 to JVF from the National Institutes of Health (National Cancer Institute). All intellectual property is owned by the Beth Israel Deaconess Medical Center. As inventor of the technology, Dr. Frangioni may someday receive royalties if products are ever commercialized.

References

1. Tucker ON, Crotty PL, Conlon KC. The management of insulinoma. Br J Surg 2006;93:264–75. [PubMed: 16498592]
2. Nikfarjam M, Warshaw AL, Axelrod L, Deshpande V, Thayer SP, Ferrone CR, Fernandez-del Castillo C. Improved contemporary surgical management of insulinomas: a 25-year experience at the Massachusetts General Hospital. Ann Surg 2008;247:165–72. [PubMed: 18156937]
3. Warner RR. Enteroendocrine tumors other than carcinoid: a review of clinically significant advances. Gastroenterology 2005;128:1668–84. [PubMed: 15887158]

4. Pedrazzoli S, Pasquali C, Alfano D'Andrea A. Surgical treatment of insulinoma. Br J Surg 1994;81:672–6. [PubMed: 8044542]
5. McAuley G, Delaney H, Colville J, Lyburn I, Worsley D, Govender P, Torreggiani WC. Multimodality preoperative imaging of pancreatic insulinomas. Clin Radiol 2005;60:1039–50. [PubMed: 16179163]
6. Guettier JM, Kam A, Chang R, et al. Localization of insulinomas to regions of the pancreas by intraarterial calcium stimulation: the NIH experience. J Clin Endocrinol Metab 2009;94:1074–80. [PubMed: 19190102]
7. Adams S, Baum R, Rink T, Schumm-Drager PM, Usadel KH, Hor G. Limited value of fluorine-18 fluorodeoxyglucose positron emission tomography for the imaging of neuroendocrine tumours. Eur J Nucl Med 1998;25:79–83. [PubMed: 9396878]
8. Fedorak IJ, Ko TC, Gordon D, Flisak M, Prinz RA. Localization of islet cell tumors of the pancreas: a review of current techniques. Surgery 1993;113:242–9. [PubMed: 8382841]
9. Ko TC, Flisak M, Prinz RA. Selective intra-arterial methylene blue injection: a novel method of localizing gastrinoma. Gastroenterology 1992;102:1062–4. [PubMed: 1537497]
10. Prinz RA. Selective intraarterial methylene blue for pancreatic and duodenal endocrine tumors. Journal of Hepto-Biliary-Pancreatic Surgery 1994;1:517–521.
11. Keaveny TV, Fitzgerald PA, McMullin JP. Selective parathyroid and pancreatic staining. Br J Surg 1969;56:595–7. [PubMed: 4183792]
12. Keaveny TV, Tawes R, Belzer FO. A new method for intra-operative identification of insulinomas. Br J Surg 1971;58:233–4. [PubMed: 4100889]
13. Tanaka E, Chen FY, Flaumenhaft R, Graham GJ, Laurence RG, Frangioni JV. Real-time assessment of cardiac perfusion, coronary angiography, and acute intravascular thrombi using dual-channel near-infrared fluorescence imaging. J Thorac Cardiovasc Surg 2009;138:133–40. [PubMed: 19577070]
14. Frangioni JV. In vivo near-infrared fluorescence imaging. Curr Opin Chem Biol 2003;7:626–34. [PubMed: 14580568]
15. Sens R, Drexhage KH. Fluorescence quantum yield of oxazine and carbazine laser dyes. J Luminesc 1981;24:709–712.
16. Troyan SL, Kianzad V, Gibbs-Strauss SL, et al. The FLARE() Intraoperative Near-Infrared Fluorescence Imaging System: A First-in-Human Clinical Trial in Breast Cancer Sentinel Lymph Node Mapping. Ann Surg Oncol. 2009
17. Gioux S, Kianzad V, Ciocan R, Gupta S, Oketokoun R, Frangioni JV. High power, computer-controlled, LED-based light sources for fluorescence imaging and image-guided surgery. Mol Imaging 2009;8:156–165. [PubMed: 19723473]
18. Hamaguchi K, Gaskins HR, Leiter EH. NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. Diabetes 1991;40:842–9. [PubMed: 1647994]
19. Abboud B, Boujaoude J. Occult sporadic insulinoma: localization and surgical strategy. World J Gastroenterol 2008;14:657–65. [PubMed: 18205253]
20. Imamura M, Hosotani R, Shimada Y. The Zollinger-Ellison syndrome: Review of recent progress in diagnosis and treatment. Journal of Hepto-Biliary-Pancreatic Surgery 1996;3:33–39.
21. Tseng LM, Chen JY, Won JG, Tseng HS, Yang AH, Wang SE, Lee CH. The role of intra-arterial calcium stimulation test with hepatic venous sampling (IACS) in the management of occult insulinomas. Ann Surg Oncol 2007;14:2121–7. [PubMed: 17431724]
22. Gordon DL, Airan MC, Suvanich S. Visual identification of an insulinoma using methylene blue. British Journal of Surgery 1974;61:363–364.
23. Gordon DL, Airan MC, Thomas W, Seidman LH. Parathyroid identification by methylene blue infusion. Br J Surg 1975;62:747–9. [PubMed: 51661]
24. Keaveny TV, FitzGerald P. Selective parathyroid staining. Lancet 1968;2:284. [PubMed: 4173718]
25. Patel PN. Methylene blue for management of Ifosfamide-induced encephalopathy. Ann Pharmacother 2006;40:299–303. [PubMed: 16391008]
26. Traynor S, Adams JR, Andersen P, Everts E, Cohen J. Appropriate timing and velocity of infusion for the selective staining of parathyroid glands by intravenous methylene blue. Am J Surg 1998;176:15–7. [PubMed: 9683125]

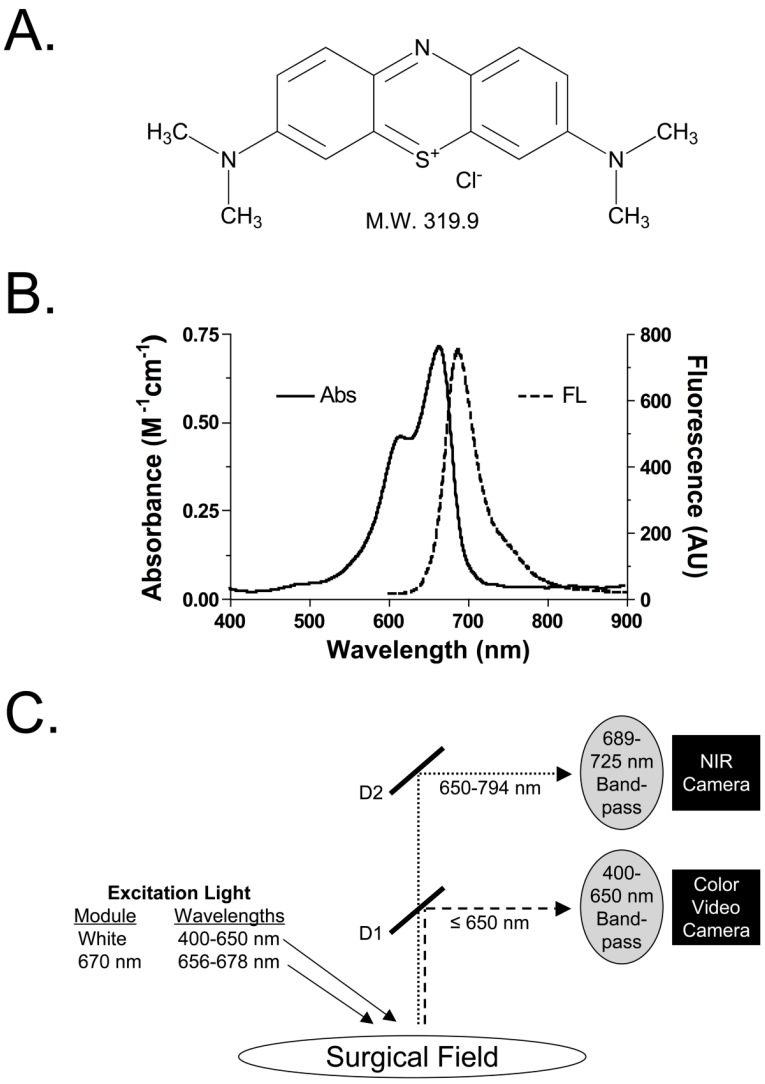

Figure 1. NIR Fluorescent Contrast Agent and Imaging System

A. Methylene blue (MB) chemical structure and molecular weight (MW).

B. Absorbance (solid curve; left axis) and fluorescence emission (dotted curve; λ_{Exc} = 655 nm; right axis) spectra of 10 μM MB in 100% serum at 37°C.

C. Schematic of the FLARE™ (Fluorescence-Assisted Resection and Exploration) intraoperative NIR fluorescence imaging system, showing excitation and emission wavelengths, and dichroic mirrors D1 and D2. Camera images are acquired and displayed simultaneously, and in real-time.

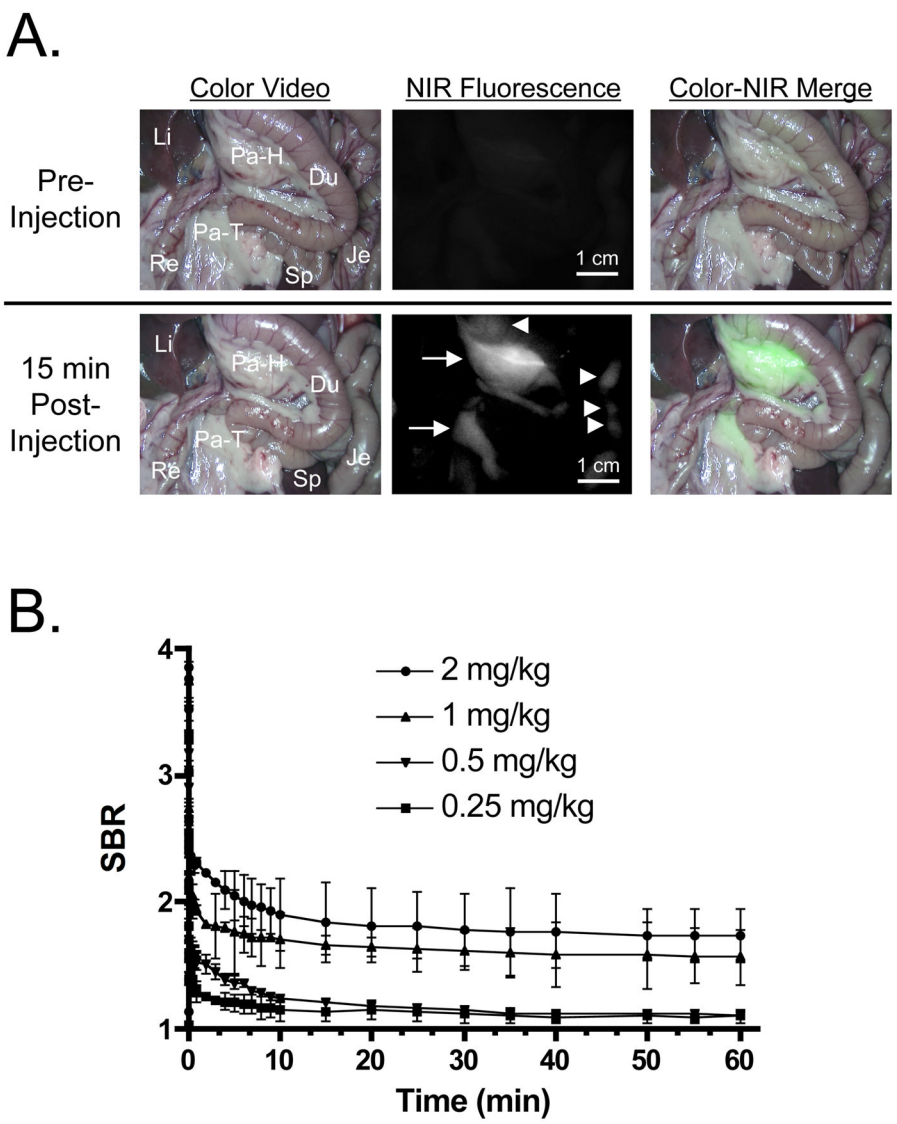

Figure 2. NIR Fluorescence Imaging of Normal Rat Pancreas Using Intravenously Injected MB

A. Representative images (n = 3 animals) of the abdominal cavity of rat pre-injection (top row) and 15 min post-injection (bottom row) of 1.5 mg/kg MB given as an IV bolus. Shown are color video (left), 700 nm NIR fluorescence (middle), and a pseudo-color (lime green) merge of the two (right). Arrows = pancreas. Arrowheads = food particles in lumen of small bowel. NIR fluorescence images were acquired with a 150 msec camera exposure time and displayed with identical normalizations. Du = duodenum; Je = jejunum; Li = liver; Pa-H = head of pancreas; Pa-T = tail of pancreas; Re = rectum; Sp = spleen.

B. Signal-to-background ratio (SBR; mean ± SEM) over time in normal rat pancreas after IV bolus injection of MB at the doses shown and a 150 msec camera exposure time. N = 3 rats per group.

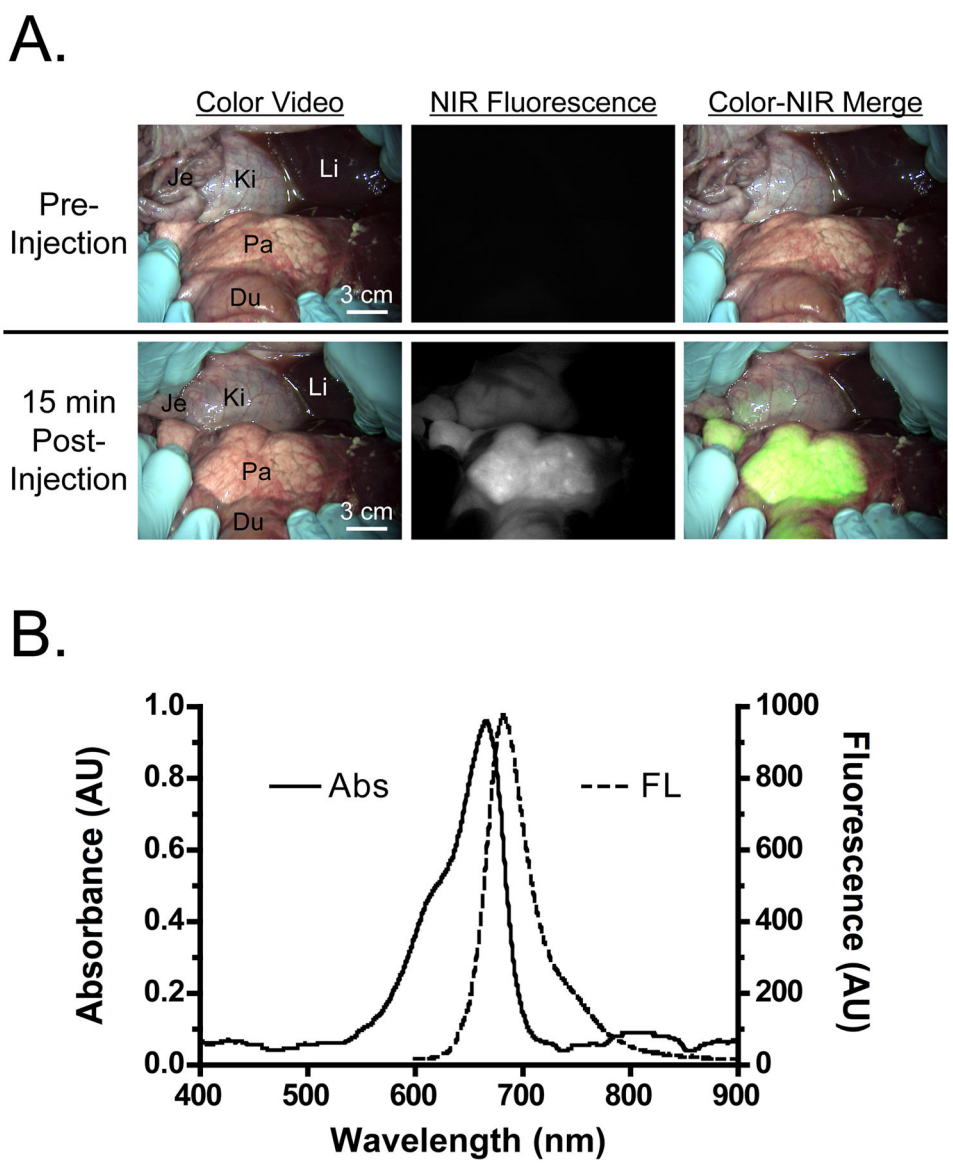

Figure 3. NIR Fluorescence Imaging of Normal Pig Pancreas Using Intravenously Injected MB

A. Representative images (n = 4 animals) of the abdominal cavity of pig pre-injection (top row) and 15 min post-injection (bottom row) of 1.5 mg/kg MB given as an IV bolus. Shown are color video (left), 700 nm NIR fluorescence (middle), and a pseudo-color (lime green) merge of the two (right). NIR fluorescence images were acquired with a 250 msec exposure time and displayed with identical normalizations. Du = duodenum; Je = jejunum; Ki = kidney; Li = Liver; Pa = pancreas.

B. Reflectance absorbance and fluorescence spectra of pig pancreas 1 h post-injection of 1.5 mg/kg MB. Fluorescence excitation was 655 nm.

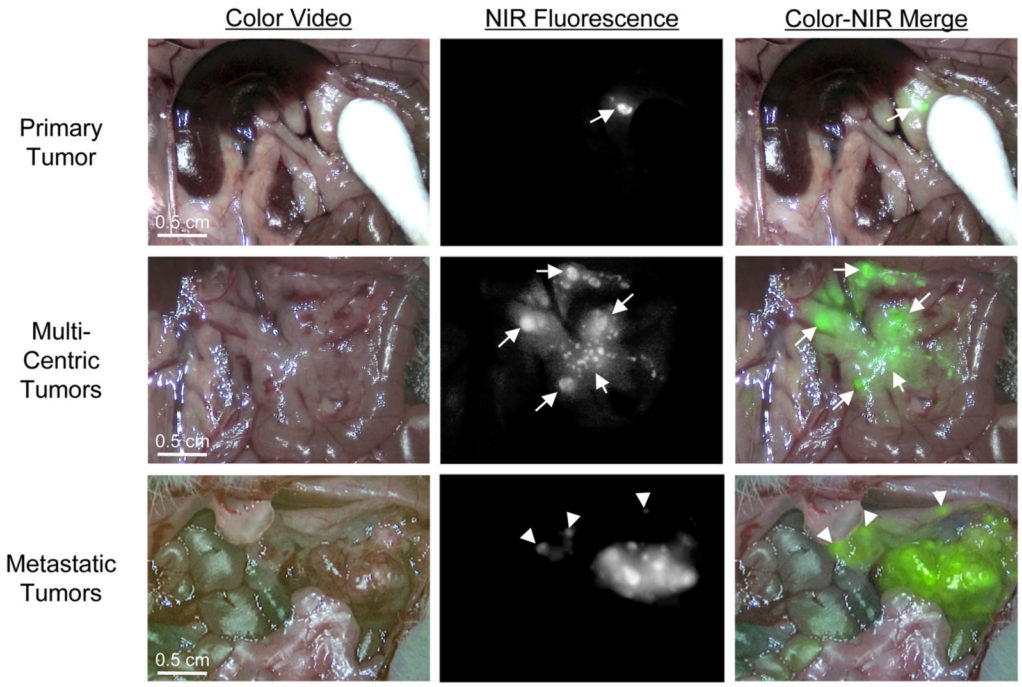

Figure 4. NIR Fluorescence Imaging of Insulinoma Using Intravenously Injected MB

Insulinoma-bearing NOD/ShiLt-Tg(RipTAg)1Lt/J mice were imaged 15 min after IV bolus injection of 1.5 mg/kg MB. Shown are color video (left), 700 nm NIR fluorescence (middle), and a pseudo-colored (lime green) merge of the two (right). NIR fluorescence images were acquired with a 150 msec exposure time and displayed with identical normalizations. Shown are animals bearing a single primary tumor (arrow) in the pancreas (top row), multi-centric pancreatic tumors (arrows; middle row), and metastatic tumors (arrowheads; bottom row).

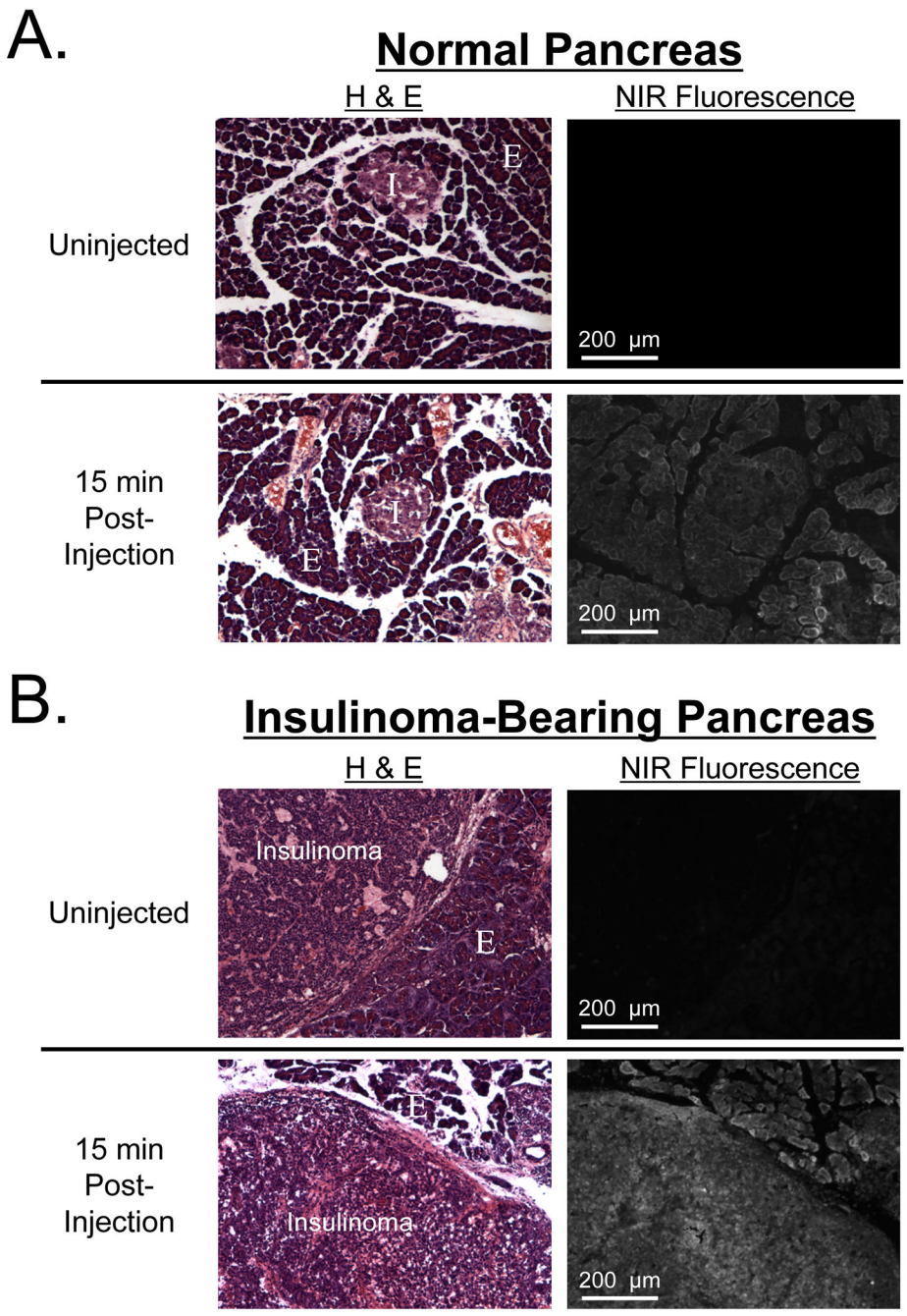

Figure 5. Histological Analysis of Normal and Tumor-Bearing Pancreas

A. Consecutive sections from normal rat pancreas stained with H&E (left) or unstained and imaged at 700 nm using a NIR fluorescent microscope (right). Rats were either uninjected (top row) or injected IV with 1.5 mg/kg MB and sacrificed at 15 min post-injection (bottom row). NIR fluorescence images have identical exposure times (5 sec) and normalizations. Results are representative of n = 3 independent experiments. I = pancreatic islets; E = exocrine pancreas.

B. Consecutive sections from insulinoma-bearing pancreas of NOD/ShiLt-Tg(RipTAg)1Lt/J mice stained with H&E (left) or unstained and imaged at 700 nm using an NIR fluorescent microscope (right). Mice were either uninjected (top row) or injected IV with 1.5 mg/kg MB

and sacrificed at 15 min post-injection (bottom row). NIR fluorescence images have identical exposure times (5 sec) and normalizations. Results are representative of n = 3 independent experiments. Insulinoma is indicated; E = exocrine pancreas.