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The development of fluorescence guided surgery for pancreatic cancer: from bench to clinic

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Abstract

Introduction: Surgeons face major challenges in achieving curative R0 resection for pancreatic cancers. When the lesion is localized, they must appropriately visualize the tumor, determine appropriate resection margins, and ensure complete tumor clearance. Real-time surgical navigation using fluorescence-guidance has enhanced the ability of surgeons to see the tumor and has the potential to assist in achieving more oncologically-complete resections. When there is metastatic disease, fluorescence enhancement can help detect these lesions and prevent unnecessary and futile surgeries.

Areas covered: This article reviews different approaches for delivery of a fluorescence signal, their pre-clinical and clinical developments for fluorescence guided surgery, the advantages/ challenges of each, and their potential for advancements in the future.

Expert commentary: A variety of molecular imaging techniques are available for delivering tumor-specific fluorescence signals. Significant advancements have been made in the past 10 years due to the large body of literature on targeted therapies and this has translated into rapid developments of tumor-specific probes.

Keywords

Pancreatic cancer; fluorescence-guided surgery; antibodies; fluorophores; mouse models

1. Introduction

Pancreatic cancer is a recalcitrant malignancy with a poor prognosis. In 2018, it is the 3rd most common cause of cancer related deaths in the United States. Approximately 12.5 new cases are diagnosed per 100,000 people per year.

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Declaration of interest

RM Hoffman declares that they are a non-salaried affiliate of AntiCancer, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. A reviewer on this manuscript has disclosed that they are one of the founders of SurgiMAb, a biotech company that develops SGM-101, a dye-antibody conjugate cited in the manuscript. Peer reviewers on this manuscript have no other relevant financial or other relationships to disclose.

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When the disease is localized, complete surgical resection to negative margins is the only chance at cure. Unfortunately achieving tumor free surgical resection is challenging. Pre-operative imaging modalities can assist in localizing the lesion. But once in the operating room, surgeons rely only on bright light visualization, tactile cues, and knowledge of anatomic boundaries, along with surgical judgment to find the lesion and to determine appropriate resection margins. Intra-operative frozen sections further assist in detecting residual cancer, but this can be affected by sampling error as it is impossible to completely survey the entire resection bed[1]. These issues lead to incomplete resections, with R1 microscopically positive margins seen in as many as 50–70% of curative intent surgeries[2–5]. This leads to an unacceptably high number of early recurrence and poor outcomes in patients undergoing curative-intent surgeries[6,7]. Tumor clearance has been shown to be an independent predictor of post-resection outcome and survival[8–10]. Contrast enhancement using a tumor-specific fluorescence signal can help surgeons visualize the lesion, determine resection margins in real-time during the surgery, and survey the resection bed after tumor removal to detect foci of residual disease. The use of fluorescence-guided surgery (FGS) for intra-operative visualization of pancreatic tumors has the potential to increase rates of oncologically complete resections.

When the disease is no longer localized, surgery is no longer an option. While most of these cases are diagnosed using conventional computed tomography (CT) or magnetic resonance imaging (MRI), up to 25% of patients undergo laparotomy only to discover radiographically occult lesions[11,12]. These lesions are usually beyond the detection of even high-resolution contrast-enhanced CT or MRI, and they can only be diagnosed by direct visualization of the abdominal cavity. Staging laparoscopy has been used to detect hepatic or peritoneal metastases and can decrease the probability of finding unresectable disease upon laparotomy from 41% to 20%[13]. The use of tumor-specific fluorescence during laparoscopy can enhance visualization of these deposits and has the potential to further improve the detection rates of disseminated disease. This can stage patients more accurately, prevent unnecessary laparotomies, and select out patients with metastases who will not benefit from local pancreatic resection.

Novel molecular imaging techniques for delivering fluorescence have been developed for intra-operative surgical guidance. An ideal probe would clearly label the pancreatic tumor, affected lymph nodes, or distant metastases with a strong contrast and spare normal tissue. The fluorescence signal gives real-time feedback to guide the surgeon in determining optimal resection boundaries, dissect appropriate lymph node basins, and enhance detection of any hepatic or peritoneal metastases that would preclude resection of the primary lesion. The present report reviews strategies for delivery and detection of fluorescence signals, pre-clinical and clinical developments, and challenges facing the use of fluorescence guided surgery (FGS) for pancreatic cancer.

The use of fluorescence imaging technology in the operating room has greatly advanced real-time in-vivo imaging. While intra-operative fluoroscopy, CT or ultrasound have advanced real-time image guidance during surgeries, the systems are limited in their use due to the prohibitive equipment, ionizing radiation, need for specialized training or user variability.

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2. Probe design for tumor-specificity

We can utilize the body of knowledge accumulated in characterizing biomarkers unique to cancer cells or the tumor micro-environment to help distinguish the neoplasm from normal parenchyma. These are based on target-ligand binding due to the nature of cancers to differentially overexpress enzymes, receptors, or antigens compared to normal tissue. Logical probes can then be designed to exploit the biology of these lesions for tumor-specific fluorophore delivery.

Some probes carry an "always-on" fluorescence signal and use a target-ligand binding to provide contrast while non-specific binding is washed out over time. These probes have a constitutively active signal regardless of tumor proximity[14]. They face challenges with off-target signal accumulation in the reticuloendothelial system, have increased background noise, and necessarily require a washout time for optimal contrast, during which the on-target binding can also diminish[15]. Therefore, target binding must be stronger than background and the must persist long enough for a detectable signal to be present after washout.

Other probes have a quenched fluorophore that becomes activated after enzymatic activity at the target site. While the activatable probes intrinsically have a lower background signal, they also face unique challenges in that a larger amount of the probe must be delivered to gain proximity to the tumor for activation and remain at the tumor without diffusing away after activation[16,17]. The unquenching reaction must additionally occur with appropriate kinetics such that a sufficient amount of fluorescence is present for a detectable signal at the time of visualization[18,19].

As the field of FGS expands, the number and sophistication of fluorophore delivery platforms will increase. The present report reviews some key pre-clinical and clinical tumor-specific fluorescent probes available for treatment of pancreatic cancer.

2.1. Activatable probes

Cancer associated enzymes have been exploited for functional activatable fluorescent probes. Some cancer associated enzymes that have been evaluated for fluorescence in-vivo imaging of solid tumors are cathepsins[20], matrix metalloproteases[21,22], β-galactosidase[23], and γ -glutamyltranferases[24]. γ -glutamyltransferase (GGT) and matrix metalloprotease (MMP) based enzyme activatable fluorescent probes have been evaluated in pancreatic cancer.

GGT is a cell surface enzyme that is present in most cells, but is frequently upregulated in GI malignancies[25]. Urano et al used the enzymatic function of GGT with γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG), a hydrophilic probe that is quenched by spirocyclic caging[24]. Upon proteolysis by GGT, the hydrophobic fluorescent HMRG product is "uncaged" and taken up into cancer cells into the area. The reaction is rapid and a fluorescence signal can be detectable within minutes of topical application. This probe has been used clinically to detect a strong fluorescence signal in a number of solid GI cancer specimen after resection including hepatocellular carcinoma, cholangiocarcinoma, and

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colorectal liver metastases with the brightest signal after 30 minutes[26]. It has also been used to detect pancreatic cancer from endoscopic ultrasound guided fine needle aspiration specimens in a small group of 10 patients[27]. gGlu-HMRG was able to macroscopically detect pancreatic cancer in several specimens, however since normal pancreatic acini also expressed GGT, the approach was not sufficiently specific. The approach has been modified to develop a chymotrypsin cleavable probe (glutaryl-phenylalanine hydroxymethyl rhodamine green [gPhe-HMRG]) to detect pancreatic leaks after reconstruction during pancreatic surgery[28]. Filter paper applied to the pancreatic stump was sprayed with gPhe-HMRG and a positive fluorescence signal indicated areas of leakage which were confirmed on further microscopic evaluation. While further development is necessary, the technique is appealing in that it does not rely on systemic administration of the probe to the patient. This approach would be limited for use in resection of localized pancreatic cancers, but can be applicable to detection of peritoneal carcinomatosis. The topical application limits the dose of the reagent while still allowing for rapid fluorescence detection within minutes.

Solid malignancies, especially pancreatic cancers have increased expression of MMP's, notably MMP2 and MMP9[29,30]. Metildi et al used this enzymatic function with ratiometric activatable cell penetrating peptides (RACPP's) conjugated to Cy5 and Cy7 fluorophores for visualization of pancreatic tumors in FGS[31]. The MMP based RACPP's have polycationic molecules joined to neutralizing polyanions with a cleavable linker and upon proteolysis, the neutralizing linker dissociates, allowing preferential uptake of the Cy5 fluorophore linked cation into cancers cells in the area[22]. RACPP's were administered to mice bearing pancreatic orthotopic xenografts and the ratio of Cy5 (cleaved, activated peptide) compared to the ratio of Cy7 (uncleaved, intact peptide) was used for intra-operative navigation and FGS. The peptide was successfully able to label pancreatic orthotopic xenografts 2 hours after injection and the use of RACPP guided FGS improved local and distant recurrence rates as well as disease-free interval compared to standard bright light surgery (BLS). Current clinical utility of RACPP's have been tested in Phase I and II clinical trials for breast cancer[32] (NCT02391194, NCT03113825).While there are no trials yet with this probe for pancreatic cancer, it will be an interesting agent for future clinical evaluation.

These activatable probes are advantageous in that the background signal is minimized, however they face drawbacks in optimizing adequate accumulation and enzymatic activation for production of a strong tumor signal.

2.2. Peptide binding

Tumors overexpress a variety of receptors and using small molecules conjugated to fluorophores to target peptide binding pockets of these receptors is another approach to introducing specificity for fluorescence in-vivo imaging. Due to their small size, peptides have more rapid pharmacokinetic properties. VanDam et al first showed clinical utility of this approach by using folate conjugated to FITC in order to approach folate receptor (FR) overexpressing ovarian cancers and later, breast cancers[33,34]. While this approach was not feasible for pancreatic cancers with approximately 10–20% overexpressing FR other peptides have been evaluated[35,36].

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Integrins are trans-membrane receptors regulating cell-extracellular matrix interactions and are highly overexpressed in a number of GI malignancies, including pancreatic cancer, making them an ideal target for molecular imaging using fluorescently labeled small peptide probes[37]. Cyclic arginine-glycine-aspartic acid (cRGD) peptides have been designed to target a number of integrins, most notably the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_6$. Liu et al conjugated cRGD to the NIR dye LICOR-IRDye800CW[38]. Handgraaf and Boonstra et al conjugated cRGD to a different NIR fluorophore ZW800–1[39,40]. Both groups successfully imaged pancreatic xenografts tumor accumulation of the probe as early as 35 minutes and as late as 24 hours. Microscopy by Handgraaf and Boonstra et al showed that the probe is internalized and that it is the continuous cycling of internalization and recycling of integrins to the surface membrane allowed for this wide window of imaging from 35 minutes to 24 hours. Clinical development of the probe has focused on PET based cRGD tracers which have completed safety and efficacy in Phase I/II trials in brain cancers, lung cancers, head and neck cancers, sarcomas, melanomas[41] (NCT00565721) and is a potentially viable probe for FGS.

Cysteine knot peptides are a further development on integrin binding, engineered with a higher binding potency. These peptides are small (~4kDa), administered at sub nanomolar doses compared to cRGD, and are highly stable in a variety of biologic, chemical and physical environments[42]. Tummers et al linked the cysteine knot peptide specifically targeting $\alpha_v\beta_6$ to LICOR-IRDye800CW and showed fluorescence labeling of subcutaneous and orthotopic pancreatic xenografts after 2 hours[43]. They further validated the probe in a transgenic mouse model and showed that it could differentiate between carcinoma and peri-tumoral inflammation even at a microscopic level. This peptide is currently in Phase I clinical trial for PET imaging (NCT02683824) and shows promise as a competitive agent for small peptide based fluorescence delivery.

Urokinase-plasminogen activator is a protease that interacts with its cell surface receptor, uPA receptor (uPAR), to mediate cell motility, ECM degradation, and metastasis. Similar to integrins, it is overexpressed in a number of malignancies including 86–90% of pancreatic cancer and correlates with poor prognosis[44]. Lee et al developed a multifunctional probe that has both imaging and therapeutic capacity by linking a peptide derived from the amino terminal fragment (ATF) of uPA to both an 830nm dye and the drug doxorubicin encapsulated within magnetic iron oxide nanoparticles (IONP)[45]. The resulting construct called NIR-ATF-IONP-Dox was injected weekly for four weeks into mice bearing orthotopic pancreatic xenografts. There was a specific fluorescence signal present over the pancreatic tumors 6 days after the last treatment, and staining of tissue sections showed drug uptake at cells at the tumor edge and tumor-stromal boundary. uPAR has been evaluated as an accurate marker for identification of pancreatic cancers along with other well-known markers such as CEA[46]. The uPAR binding peptide ATF is a unique molecular probe with versatile applications.

At 4–17kDa, the reviewed peptides are lightweight and have rapid pharmacokinetics for fluorophore delivery. They can penetrate tumors quickly to produce a fluorescent signal within several hours. However, a drawback of these small molecules is their limited contrast and a renal elimination pattern. The tumor-to-background ratio (TBR) or signal-to-noise

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ratio (SNR) of these probes is in the range of 2–5. Additionally, the renal elimination leads to a fluorescence signal intensity at the kidneys and bladder that is often stronger than that of the tumor. For most clinical applications of FGS GI malignancies, the off-target signal will not interfere with fluorescence navigation, but in retroperitoneal areas adjacent to the kidneys this can potentially be an issue.

2.3. Antibody antigen binding

Antibodies are extraordinarily specific probes for development in FGS and the most generalizable platform for fluorophore delivery. They have been used to stain for cellular and subcellular structures in tissue for many years in pathologic diagnoses and in bench top research. It is only in the past 10 years that we have utilized this same approach in-vivo for immunodetection. With the increasing number of FDA approved therapeutic antibodies, and an increasing interest in using them for deliver chemotherapeutics, there is a tremendous body of information on development, engineering, pharmacokinetics, pharmacodynamics, safety and efficacy, in conjugation of these molecules. There are several promising markers specific to pancreatic cancer that have been evaluated for FGS. These are the human carcino-embryonic antigen (CEA), sialyl-Lewis antigen A also known as cancer antigen 19–1 (CA19–9), and cell surface associated mucin 1 (MUC1).

CEA was the first antigen-antibody target used to first describe in-vivo tumor-specific fluorescence in 1991[47]. It is a surface glycoprotein involved in cell-adhesion and motility that is expressed in embryonic tissue, not present in normal adult tissue, and is reactivated in cancers, especially GI solid tumors[48]. Pèlegrin et al successfully used an anti-CEA antibody linked to fluorescein and I-125 to visualize subcutaneous colorectal carcinoma xenografts in mice and demonstrated a clear fluorescence signal with radiotracer uptake of more than 30% of the injected dose at 24 hours[47]. The following year, Folli et al showed clinical proof of concept with this probe in 6 patients undergoing surgical resection for colorectal carcinoma and saw that 100% of patients had a positive fluorescent signal at the tumor which was 10× higher than normal tissue on radiotracer quantification[47,49].

Serum levels of CEA are elevated in 40–70% of patients with pancreatic cancer[50–52] and up to 84–98% of pancreatic cancers stain positive for CEA on immunohistochemistry[53–55]. Our laboratory has worked extensively with anti-CEA antibodies for fluorescence in-vivo imaging of pancreatic cancers. Kaushal et al first demonstrated in 2008 the efficacy of a murine anti-CEA antibody conjugated to visible wavelength dyes (AlexaFluor488 or Oregon Green) in labeling orthotopic and peritoneal dissemination mouse models of pancreatic cancer[56]. The fluorescence intensity was dose dependent, up the maximal 75 μg dose tested and the highest signal intensities were obtained at 24 and 48 hours. Metildi et al use this probe with AlexaFluor488 to further show feasibility in laparoscopic detection of submillimeter peritoneal deposits[57]. Metildi et al further showed improved local disease control and a survival advantage in using this fluorescent anti-CEA antibody in a direct comparison between FGS and BLS[58]. Mice that underwent FGS had a 92% rate of complete resection vs. 45.5% in BLS and this correlated with cure rates (40% FGS vs 4.5% BLS) and 1 year survival rates (FGS 28% vs. 0% BLS). Hiroshima et al used a chimeric anti-CEA antibody conjugated to DyLight650 to evaluate FGS in a pancreatic PDOX model

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and showed that there was tumor-specific fluorescent labeling with a 50ug dose 24 hours after injection. Neoadjuvant chemotherapy in conjugation FGS using this probe showed superior outcomes in local recurrence and development of metastatic disease[59]. Maawy et al further evaluated this chimeric anti-CEA antibody with a number of fluorophores (DyLight 488, 550, 660, 755, AlexaFluor 488, 555, 660, 750) and found that while the shorter wavelength dyes were more photostable over time, the longer wavelength dyes, especially those close to the NIR range had improved tissue penetration and decreased auto-fluorescence[60]. We have since now evaluated a humanized version of the anti-CEA antibody conjugated to the LICOR-IRDye800CW and demonstrated clear localization in pancreatic cancer with the highest fluorescence intensities at 24 and 48 hours and strong contrast at tumor-to-background ratios of up to 16.6 at 48 hours after a 75ug dose[61]. This humanized anti-CEA antibody called h-M5A has completed Phase I/II clinical trials for safety and efficacy and is currently being evaluated for immuno-PET imaging and radioimmunotherapy in GI malignancies including pancreatic cancer (NCT02293954, NCT00645060).

SGM-101 is a chimeric anti-CEA antibody conjugated to a BM104, a 700nm NIR fluorophore. It is an antibody-based probed that has undergone the most extensive clinical development and evaluation for FGS. Gutowski et al showed that the probe was able to label orthotopic mouse models of colon and pancreatic cancer, with a TBR of 3.5 at 48 hours after a 30ug injection[62]. The probe has since undergone Phase I/II clinical trials for intra-operative fluorescence detection of colorectal and pancreatic cancers as well as peritoneal carcinomatosis due to GI malignancies (NCT02784028, NCT02973672) and plans for a multi-center Phase III clinical trial are underway.

Other relevant anti-CEA antibodies under clinical evaluation for pancreatic cancer are KAb201, a chimeric anti-CEA antibody, for immuno-PET imaging in the UK[63] (SRCTN 16857581) and hMN14 also known as labetuzumab, a humanized anti-CEA antibody, tested for radioimmunotherapy[64] (NCT00041639). The number of competing anti-CEA antibodies that have been developed highlighting the intense interest and potential of CEA as a target.

CA19–9 is a surface glycoprotein expressed on surface of cancer cells due to aberrant sialyl transferase during carcinogenesis[65]. Serum levels are elevated in over 70% of patients with pancreatic cancer and levels of the antigen are used to follow disease progression and correlate with outcome. Up to 94% of pancreatic cancers stain positive for CA19–9 on immunohistochemistry[66]. Our group first described the use of an anti-CA19–9 antibody linked to a visible wavelength dye AlexaFluor488 and showed a linear dose dependent tumor accumulation of fluorescence signal up to 100ug in orthotopic and peritoneal dissemination mouse models of pancreatic cancer[67]. While, there was a strong tumor signal at 24 hours and a peak at 48 hours after a 75ug administration, continued imaging of the mice up to 3 weeks showed persistence of a detectable fluorescence signal. Fluorescence microscopy showed that the probe was specific to the tumor cells and not the stroma. Hiroshima et al used the anti-CA19–9 antibody conjugated to DyLight650 in a CA19–9 high, CEA-low PDOX mouse model to show that a combination of neoadjuvant chemotherapy along with FGS was necessary especially to address local recurrences and

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metastatic disease[68]. A fully human version of the anti-CA19–9 antibody called Humab5B1 has been developed. The parental antibody has completed its Phase I clinical trial, and conjugates for immune-PET and radioimmunotherapy are currently undergoing Phase 1 trials in pancreatic cancer and other CA19–9 positive GI cancers (NCT02687230, NCT02672917, NCT03118349). Minor drawbacks to consider are the increased levels of CA19–9 during pancreatic inflammation and biliary obstruction which could lead to a falsely positive fluorescence signal over non-neoplastic tissue[69]. Additionally, 10% of patients lack the Lewis antigen on their red blood cells and will not express the this marker even with a large tumor burden[70]. Despite these, CA19–9 has a wide application for most pancreatic cancers and is also an appealing target for FGS.

Mucins line the epithelial surfaces of epithelial cells and MUC1 is a member of the family of cell surface glycoproteins. MUC1 is normally expressed over the apical surfaces of epithelial cells, but in pancreatic cancers the protein is aberrantly glycosylated and overexpressed on the basolateral membrane[71]. Its aberrant overexpression is seen in over 90% of pancreatic cancers[72]. Park et al use an anti-MUC1 antibody conjugated to DyLight 650 or 550 fluorophore and showed tumor-specific labeling in orthotopic pancreatic xenografts mouse models[73]. Flow cytometry indicated that only 24.4–36.9% of the pancreatic cancer cells evaluated express MUC1. Despite this there was still a TBR of 2.4–6.7 at 7–10 days after a 30ug dose on whole body in-vivo imaging. Humanized anti-MUC1 antibodies such as hPankoMab and cantuzumab (huC242) have been evaluated in a number of clinical trials for targeted drug delivery, immunotherapy and radio-immunotherapies for pancreatic cancer (NCT02587689, NCT00352131), but the use of MUC1 antibodies for FGS has not been as thoroughly developed[74,75].

Clinically available therapeutic antibodies have been adapted with fluorophores for use in FGS. While these drugs are not necessarily indicated for treatment of pancreatic cancer, there is still a differential expression of the target antigen compared to normal tissue such that can be exploited to deliver a contrasting fluorescent signal for intra-operative imaging. With pre-existing in-human safety and bio-distribution profiles, novel antibody fluorophores conjugates can undergo more rapid clinical translation. The development fluorescent anti-epidermal growth factor receptor (EGFR) antibodies and anti-vascular epidermal growth factor (VEGF) antibodies are clear examples of this progress.

Cetuximab is a chimeric anti-EGFR antibody that is used as a targeted therapy for head and neck cancers, metastatic colon cancers, and EGFR positive non-squamous cell lung cancers (NSCLC). EGFR is overexpressed in over 90% of pancreatic cancers and therapies inhibiting EGFR have shown some efficacy, but it is not a primary treatment option for this disease[76]. Cetuximab was conjugated with fluorophores for FGS in head and neck cancer in by Rosenthal et al in 2006[77]. They showed that a 50ug dose of Cetuximab-Cy5.5 could clearly label orthotopic head and neck squamous cell cancer (HNSCC) xenografts, with strong signals from 24–72 hours. They further combined a number of available therapeutic antibodies (bevacizumab, cetuximab, panitumumab, trastuzumab, and tocilizumab) to IRDye800CW and showed feasibility in imaging head and neck cancers as well as breast cancers[78,79]. These fluorescently labeled antibodies were successfully imaged in both a small animal fluorescent imaging device specific for IRDye800CW (Pearl Impulse, LI-COR

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Biosciences, Lincoln, NE) and a clinically available fluorescent imaging device designed for ICG which shares the 800nm wavelength (Spy Elite, Novadaq Technologies, Mississauga, Canada). The work demonstrated the utility of repurposing a clinically available NIR fluorescent imaging system with an overlapping spectrum[80]. Rosenthal et al first evaluated safety and feasibility of the cetuximab-IRDye800CW probe using doses ranging from 1% –25% of the therapeutic dose in patients with head and neck SCC (NCT01987375) and saw that the best fluorescent signals were obtained in patients who received, 10–25% of the therapeutic dose which translated into 10–25 mg/m^2 of the probe[81]. The phase I trial of cetuximab-IRDye800 has been completed and the evaluation of cetuximab-IRDye800CW was expanded to pancreatic cancer in a Phase II clinical trial in 2016 (NCT02736578)[82]. Panitumumab, a fully human version of the anti-EGFR antibody, conjugated to IRDye800CW is also being evaluated for pancreatic cancer in a Phase I/II trial that opened at the end of 2017 (NCT03384238).

Bevacizumab is a humanized anti-VEGFa antibody that is used as a targeted therapy for colon cancer, lung cancer, glioblastoma, and renal-cell carcinoma. Similar to cetixumab, it is not used as a primary treatment for pancreatic cancer, but overexpression of VEGF is seen in 64–73% of pancreatic cancers[83,84]. Withrow et al first used bevacizumab conjugated to Cy5.5 to image subcutaneous head and neck SCC xenografts[85]. Using a 165ug dose and imaging at 48 hours post-injection, they were able to visualize the lesion for FGS, with a sensitivity of 80.9% and a specificity of 91.7% for detection of residual disease. It was evaluated in pre-clinical models of melanoma, breast, colon, and pancreatic cancer[78,79,86,87]. Bevacizumab conjugated to IRDye800CW has been evaluated for patients with colon cancer peritoneal metastases undergoing cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC)[88] (NTR4632) and is currently undergoing Phase I/II trials for intra-operative visualization of pancreatic cancer (NCT02743975).

Antibodies that are unique to pancreatic cancer can be targeted along with fluorophores for FGS. Such antibody fluorophore conjugates are promising for development in FGS and immunoimaging in general, but they are still in nascent stages of development, making their way through early clinical trials. Evaluating preexisting therapeutic antibodies out in clinical use is another approach. Studies with fluorophore conjugated cetuximab and bevacizumab indicate that although the drug is not primarily used for treatment in pancreatic cancer, as long as there is a tumor specific upregulation of the target, tumor-specific fluorescent labeling can still be achieved for intraoperative imaging. Additionally, the dose of the fluorescent antibody needed to obtain a sufficient fluorescent signal can be a fraction of the therapeutic dose, leading to an even greater safety profile.

Antibodies confer remarkable specificity and their larger molecular size leads to improved serum retention and higher overall signal intensity compared to small molecule based probes[15]. Their Fc region and FcRn recycling further increase serum half-life. At 150–180kDa in size, these large, bulky, “always-on” probes take several hours to detect a signal at the tumor, and even longer, up to 1–2 days, for the background to wash out and achieve optimal contrast. To overcome the issue of molecular size, approaches for engineering antibodies into smaller fragments such as Fab, minibodies, diabodies, and signal chain

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variable fragments (ScFv) have been explored[15]. There are many molecules that have been derived from their parental antibodies and it is beyond the scope of this article to discuss them, but these intermediate to small probes (25–80 kDa) generally face challenges in stability, tend to aggregate, and rapidly clear from the serum. They have been evaluated for immuno-imaging and radio-immunotherapies, but have not been used frequently for FGS.

Even smaller classes of antigen binding molecules or alternative scaffolds, such as nanobodies (15kDa) and centyrins (10kDa) conjugated to fluorophores are being evaluated[89–92]. These smaller lightweight antibody-mimics have been engineered with greater stability, while retaining the specificity and three-dimensional binding of antibodies[93,94]. They can penetrate tumors more rapidly and more effectively; even lesions with high interstitial pressure such as pancreatic cancer. Fluorescence probes with these molecules are early on in their development. While they have shown promise in demonstrating tumor-specific signal detectable at within 1–3 hours, they also clear rapidly from the serum and show a renal elimination pattern with strong fluorescence at the kidneys, a challenge common to all small molecule probes.

To address the background signal that is inherent in “always-on” probes, approaches to make antibodies activatable by quenching the fluorophore until internalized and activated in an acidic endosomal-lysosomal compartment have shown some promise[95,96]. These are also very early in development and further studies are needed to evaluate their potential for FGS.

3. Fluorophore selection and Near-Infrared Wavelengths

The feasibility of tumor-specific in-vivo fluorescence delivery for pancreatic cancer has been demonstrated in studies, some of which are discussed above. Most of these evaluated probes have been conjugated to fluorophores in the visible wavelength. This is useful for visualization of surface lesions and convenient for workflow since the signal is detectable without the assistance of a dedicated camera interface. This however becomes a critical limitation as issues of high autofluorescence and poor depth penetration challenge visible wavelength fluorophores. The quantum yield of exciting photons are affected by absorption and light scattering of biomolecules such as hemoglobin and melanin as well as aromatic amino acids and certain vitamins[97]. Endogenous fluorophores lead to emission of an autofluorescence signal in the 450–650 nm range which interfere with detection of a true signal[98]. Development of targeted fluorophores in the near-infrared (NIR) range (750–900nm) have greatly advanced the clinical use of fluorescence for real-time in-vivo imaging[99]. More recently developed probes preferentially use NIR fluorophores due to this issue. The only two currently FDA approved NIR-fluorophores are methylene blue (MB) and indocyanine green (ICG).

3.1. Methylene Blue

Methylene blue has traditionally been used at high concentrations as a blue dye for sentinel lymph node mapping under bright light, but at low doses can emit a moderate fluorescence signal when excited at 700nm. It has sporadic uptake in certain cancers and has been used to evaluate breast cancers and parathyroid glands, but the contrast is limited[100,101]. In pancreatic surgery, the use of MB fluorescence was limited to pre-clinical models to

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visualize insulinomas and one case report discussing its use in visualizing a solitary fibrous tumor of the pancreas [102,103].

3.2. Indocyanine Green

ICG has had broader applications in FGS and oncologic applications. ICG is a versatile reagent that was initially approved for fundoscopy and evaluation of cardiac and hepatic function[104]. The dye binds to plasma albumin and emits a strong fluorescence signal when excited with near infrared light at 800nm[105]. ICG undergoes hepatic metabolism and is excreted by hepatocytes into the biliary system. Its albumin binding properties allow ICG to remain in circulation to be used for a number of tissue perfusion studies including bowel anastomoses, vascular reconstructions, plastic surgery flaps and lymphatic mapping[106–110]. Its hepatic excretion properties make it a useful contrast agent for evaluating biliary structures[111]. ICG has been observed to have preferential uptake by some tumors such as colorectal metastases and HCC due to an enhanced permeability and retention effect, unfortunately pancreatic cancers do not take up ICG contrast[112,113]. Hutteman et al administered 5 or 10mg of ICG to patients undergoing pancreaticoduodenectomy and found that in 7/8 there was no useful dye uptake or signal localization at the tumor[114]. Larger doses of ICG at 5mg/kg have been administered to patients 24 hours prior to surgical resection of their pancreatic cancer and this shows some potential; 4/4 patients had a fluorescent signal at the tumor with a mean TBR of 4.9[115]. ICG fluorescence for pancreatic surgeries has also been applied to visualize reconstructed biliary structures, check perfusion of bowel anastomoses or identify occult hepatic metastases by negative staining[116–118].

These NIR dyes improve tissue depth penetration and have found useful clinical applications compared to visible wavelength fluorophores. However pancreatic cancer with its limited perfusion and dense desmoplastic stroma presents a unique challenge for non-specific NIR fluorophores. While they are sensitive, they are not specific. Non-targeted dyes have higher levels of false-positive and false-negative signals and cannot differentiate tumor normal tissue. Therefore tumor-specific targeting moieties conjugated to deep tissue penetrating fluorophores such as those discussed above must be utilized for optimal FGS of pancreatic cancer.

4. Imaging devices

The sensitivity of imaging technology has a large impact on signal detection of delivered fluorophore. While it is beyond the scope of this work to discuss the number of imaging devices in detail, important concepts are briefly reviewed here. Components of fluorescence imaging devices to be considered are: Excitation light source, emission filters, collection optics, signal detector, interface device, signal registration, and display format.

4.1 Excitation light source

The goal of the excitation light source is to direct only the wavelengths absorbed by the fluorophore and minimize all other sources of fluorescence including those in the emission band. The most commonly used excitation light sources are the following: white light lamps

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with filters for appropriate excitation wavelengths, light-emitting diodes (LED's), and laser diodes.

White light lamps have the lowest efficiencies since light is emitted in the broadest spectrum and only the necessary wavelength is filtered out and used. LED's have a narrower spectrum ~10 nm and while their power requirement is low, their light output is relatively low, requiring a large number of LED's to be integrated together[119]. Development of high powered LED's has helped to address this issue. Laser diodes have the most narrow spectrum ~1–2 nm thus enabling the highest efficiency[120]. This excitation light source delivers the highest fluorescence signal as it maximizes the greatest amount of incident excitation light. This also enables the highest sensitivity since background noise due to back scattered excitation light is lowest. However there can be concerns about eye and skin exposure safety and cost with the use of increasingly higher powered lasers. Most commercially available intra-operative fluorescence imaging devices use either laser or LED excitation light sources. Novadaq SPY, DaVinvi firefly, Fluobeam, IC-View are laser based fluorescence imaging devices while FLARE, Photodynamic eye, Stryker Aim are LED based.

4.2. Emission filters

Emission filters allows passage of the wavelengths emitted by the fluorophore and blocks other signals outside this indicated band. The filters need to strike a balance in optimizing signal brightness and contrast. An emission filter with a wide band will maximize signal collection and detection of lower concentrations of fluorophores, but at the cost of increasing autofluorescence and decreasing contrast.

4.3. Collection optics

The optical components that deliver the image to the signal detector must be considered. The field-of-view is dependent on the setting in which the instrument will be used: a wide-field in an open laparotomy setting or a narrow one for a laparoscopic setting. The depth of field and operating distance determine the optimal distance from the target that will result in the sharpest possible image. This may additionally vary depending on the magnification required to perform the procedure.

4.4. Signal Detection

The most common sensors used in devices are charge-coupled-devices (CCD's). However these camera chips have a low quantum efficiency especially in the near-infrared (NIR) range. Modifications of CCD's into electron-multiplied CCD's (EMCCD's) and intensified CCD's (ICD's) have increased signal detection, but at a cost of increased background noise. The operating room has many different light sources that contribute small but potentially significant signals in the emission spectra. With an especially sensitive detector, this can be a potentially significant noise that dilutes signal detection. Complementary metal-oxide semiconductor (CMOS) cameras and scientific-CMOS sensors are now emerging in fluorescence detection devices due to their improvements in high read-out rate, high bit depth, the compact size, light weight, and low read-out noise.

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4.5. Interface device

The interface is the device housing that the surgeon interacts with. These are usually either in the open laparotomy vs. minimally-invasive laparoscopic formats. Whether the devices are situated in carts, wall mounted, handheld portable systems, or within laparoscopic towers, each must be adapted for their respective use. While carts and wall mounted systems confer stability while handheld portable systems allow flexibility to visualize complex three-dimensional tissue geometry.

4.6. Signal registration and Display format

Signal registration integrates images obtained under fluorescence and white-light visualization. It produces a composite image that has both fluorescence signal and normal anatomic topography. The software overlays these images precisely so that surgeons can see the integrated and enhanced view, displayed in real-time.

5. Expert commentary

A critical issue in the surgical treatment of pancreatic cancer is the difficulty in intra-operative visualization of the lesion and detection of any peritoneal metastases. A high number of curative-intent pancreatic surgeries are seen with early recurrence at the surgical site or at distant sites, indicating the weakness in detection of peritoneal disease and the challenge in obtaining truly negative oncologic margins. The use of real-time intraoperative fluorescence imaging has the potential to improve this problem[1]. Fluorescence-guided diagnostic laparoscopy with tumor-specific probes can enhance contrast between normal and cancerous tissue, allowing detection of radiographically occult lesions and improve staging of the disease[121]. During resection of the lesion, tumor-specific fluorescence guidance can aid the surgeon in determining appropriate boundaries for resection such that revision of margins can be decreased. The technology can then be used to evaluate appropriate lymph node basins for resection. After removal of the pancreatic cancer and prior to reconstruction, the resection bed can be further surveyed with fluorescence to further assess tumor clearance and identify any areas to be biopsied for closer examination. After reconstruction, a perfusion based non-specific fluorescent dye in a different wavelength can be used to confirm adequate perfusion of anastomoses, evaluate for biliary leaks, or even pancreatic leaks with an enzyme based probe. In each step of surgery for pancreatic cancer, there are valuable applications for fluorescence guidance. Considerations for probe selection will be affected by the type of surgery performed. While non-specific noise from the liver and kidneys will not be an issue for procedures in the head and neck, or extremities, liver signals can impact visualization of pancreatic head lesions and kidney background can overwhelm detection of distal pancreatic lesions.

Developing effective tumor-specific probes is a major challenge, as there are tremendous numbers of approaches to conferring specificity for fluorophore delivery. There are advantages and disadvantages unique to each probe design, some of which have been discussed in detail above. The ideal fluorescent probe would be efficient to synthesize, have rapid pharmacokinetics and localization, have high sensitivity and specificity for tumor detection. There is significant heterogeneity in probe design, fluorophore selection, dosing,

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time of imaging and imaging devices. All these variables make it difficult to agree upon a definition of what constitutes an "adequate" signal for surgical navigation. Pre-clinical models, especially those that utilize orthotopic or in-situ tumor xenografts with detailed information on dosing, rote of administration, timing of imaging and imaging devices are a good start, but more information from in-human clinical trials are necessary to help further answer this question.

There is also a need for modifications to the approach based on patient's tumor biology. While most of the targets discussed above have been selected for overexpression in pancreatic cancers, the degree of expression in patients is variable. The minimal number of antigen per cancer cells and a minimal number of cells needed to detect an optical fluorescence signal is unknown. There are patients with tumors that may not express the target marker at the serum level or at the tumor level at a level sufficient for FGS. It is also possible that patients who do not have elevated serum levels of the antigen can still adequately express the membrane bound antigens at the tumor, sufficient for fluorescence guidance. While confirmation with pre-operative histologic evidence of tumor marker binding would be ideal, it is not possible for most patients. Boogerd et al has shown that serum levels of CEA correlate with the degree of tumor expression of CEA in pancreatic cancer (n=20) on IHC with a serum cutoff of 3ng/mL, but there was no correlation for rectal cancer (n=35)[122]. Work by Prince et al suggests that a minimum of 24,000 SCC cells incubated with a 8.6×10^{-2} μM concentration of cetuximab-IR800 was necessary for detection with no overlying tissue coverage, in a close-field animal imaging system optimized for 800nm.

Conversely, in patients who have high serum levels of the target, there is the likelihood that most probes would bind to the target in the serum, leaving little remaining reagent to bind at the tumor. Moore et al suggests that a "cold" unlabeled dose of cetuximab prior to infusion of the labeled antibody could be a solution to this: they report an increase in fluorescence intensity (32.3 vs. 9.3 relative fluorescence units) and contrast (TBR 5.5 vs. 1.7) when patients were pre-treated with unlabeled antibody[123].

Additionally, there is the possibility of antigenic shift when patients are treated with neoadjuvant chemotherapy. In patients, a decrease in serum levels of antigens such as CEA and CA19–9 indicate a treatment response, but does this correlate to a decrease in CEA and CA19–9 expression at the tumor such that fluorescence labeling of these targets during surgery is no longer an option? If so, what are the appropriate tests to determine this? Work by Hiroshima et al discussed above indicates that a positive fluorescence signal using a fluorophore-conjugated antibody was still present after neoadjuvant chemotherapy, even with a reduction of ~20–30% of cancer cells expressing the antigen[59]. In patients with an even more drastic response to neoadjuvant chemotherapy, it is possible that a cocktail of fluorescently labeled probes may be necessary to address this issue.

There is a rich diversity of platforms for in-vivo tumor-specific fluorescent labeling and FGS. These are needed to answer a tremendous number of questions as we transition from pre-clinical studies to in-human trials. A major issue is the determination of a guideline for the definition of an adequate fluorescent tumor signal and all the parameters concerning this

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measurement. The variability in patient tumor microenvironment adds a further level of complexity in this field. However, the use of FGS for oncologic resections is a tremendous shift in surgical approach with tremendous potential for improving patient outcomes. There will be rapid advancements in these areas with key issues addressed as further studies are undertaken.

6. Five-year view

The field of tumor-specific FGS is relatively new, most clinical studies in are only in Phase I/II trials right now. Significant advancements have been made in the past 10 years due to the large body of literature on immuno-imaging, radio-immunotherapies, and antibody based targeted therapies. This has led to rapid translation into clinical trials. As the field matures and the body of knowledge accumulates, more information on the threshold of detection will emerge: quantification of antigen copy number on a given cell, number of cancer cells expressing the antigen, cell density, efficacy of probe delivery, uptake/internalization, degree of overlying tissue coverage, fluorophore intensity, and the sensitivity of the detection hardware is necessary. Surgeons using FGS will need to better define characteristics of an “adequate” fluorescence signal for given procedures for various probe and device combinations. This will lead to determination of acceptable thresholds for positive and negative predictive values for probes undergoing clinical testing and give greater evidence-based insight into the broader issue of value, utility, and impact of FGS on patients. Further sophistication in probe development will lead to multi-functional targeting domains that can be both therapeutic and diagnostic. These “theranostic” probes can carry immuno-imaging agents for enhanced pre-operative localization, fluorophores for intra-operative guidance or photo-immunotherapies, and cytotoxic drugs for post-operative treatment of undetectable microscopic disease.

Fluorescence imaging is now available in many operating rooms and surgeons are becoming familiar in integrating the technology into their practices. While tumor-specific FGS can never replace clinical judgment, this is a technology that has the potential greatly enhance intra-operative decision making, improve rates of complete surgical resections, and subsequently patient outcomes.

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Reference annotations

* Of interest

** Of considerable interest

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Key issues

- A critical issue in the surgical treatment of pancreatic cancer is the difficulty in intra-operative visualization of the lesion and detection of peritoneal metastases.
- The use of real-time intraoperative fluorescence imaging has the potential to contribute to solve this problem.
- Significant advancements have been made in the past 10 years due to the large body of literature on immuno-imaging, radio-immunotherapies, and antibody-based targeted therapies
- The field of tumor-specific fluorescence guided surgery (FGS) is relatively new, most clinical studies in are only in Phase I/II trials right now.
- As the field matures and the body of knowledge accumulates, more information on the threshold of detection will emerge: quantification of antigen copy number on a given cell, number of cancer cells expressing the antigen, cell density, efficacy of probe delivery, uptake/internalization, degree of overlying tissue coverage, fluorophore intensity, and the sensitivity of the detection hardware is necessary.
- Surgeons using FGS will need to better define characteristics of an “adequate” fluorescence signal for given procedures for various probe and device combinations.
- This will lead to determination of acceptable thresholds for positive and negative predictive values for probes undergoing clinical testing and give greater evidence-based insight into the broader issue of value, utility, and impact of FGS on patients.
- While tumor-specific FGS can never replace clinical judgment, this is a technology that has the potential greatly enhance intra-operative decision making, increase rates of complete surgical resections, and subsequently patient outcomes.