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Current status and future perspectives of fluorescence-guided surgery for cancer

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Summary

Curative cancer surgery is dependent on the removal of all tumor and metastatic cancer cells. Preoperative imaging, intraoperative inspection and palpation, and pathological margin confirmation aid the surgeon, but these methods are lacking in sensitivity and can be highly subjective. Techniques in fluorescence-guided surgery (FGS) are emerging that selectively illuminate cancer cells, enhancing the distinction between tumors and surrounding tissues with the potential for single-cell sensitivity. FGS enhances tumor detection, surgical navigation, margin confirmation, and in some cases can be combined with therapeutic techniques to eliminate microscopic disease. In this review, we describe the preclinical developments and currently used techniques for FGS.

Keywords

Fluorescence-guided surgery (FGS); fluorophore; fluorescent proteins; photoimmunotherapy; theranostics

Introduction

Curative cancer surgery is designed to find and remove every cancer cell. An enormous amount of pre-surgical workup goes into patient selection to ensure the morbidity of the surgery is worth the chance for potential cure. Computerized tomography (CT), magnetic resonance imaging (MRI), ultrasonography (US), and endoscopy all aid in the staging process but their intraoperative utility is limited. By and large the surgeon must subjectively distinguish tumor from normal based on visual and tactile contrast between diseased and unaffected tissue [1]. An R0 resection, defined as a microscopically negative margin, is paramount for achieving a cure and long-term survival for the surgical cancer patient [2,3]. The question that every patient and practitioner wonders is, "how do you know if you got it all?" and up until recently the answer was some form of "we don't know for sure."

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New techniques are emerging that are revolutionizing the way we see and perform cancer surgery [4]. Intraoperative fluorescence imaging, or fluorescence-guided surgery (FGS), can provide high fidelity tumor visualization for real-time localization, resection, and margin confirmation in cancer surgery [5]. Unlike computerized tomography and positron emission tomography, FGS is performed without the use of ionizing radiation. Nguyen *et al.* reviewed targeted fluorescent labeling of cancer, forecasting a paradigm shift in the way we find and treat cancer [6]. In another review, Vahrmeijer *et al.* discussed clinical and preclinical applications for FGS [7]. Since these reviews a number of notable advances have been made. Here we will provide an overview of the latest preclinical advances, current clinical uses, challenges and shortcomings, as well as the future directions and applications of fluorescence-guided surgery and laparoscopy in the clinical treatment of cancers.

Development of Fluorescence Laparoscopy

With new techniques emerging to fluorescently label tumors it has been necessary to develop new systems to integrate this technology into the operating room. Laparoscopic surgical systems have recently been enhanced with a fluorescence excitation light that enables imaging of fluorescently labeled tumors and metastases as well as the surrounding anatomy in orthotopic mouse models of cancer [8]. The ideal system would maximize the fluorescent intensity of tumor tissue, minimize background fluorescence, and maintain the ability for the surgeon to visualize surrounding tissues to allow for spatial orientation and surgical navigation.

Our group developed a fluorescence laparoscopy model with the use of a xenon light source and specialized excitation and emission filters that allowed for real-time localization of fluorescently labeled tumors in the abdomen of mice [9]. With the proper filters, the sensitivity and accuracy of staging laparoscopy were significantly improved, allowing for the detection of tumor deposits that were less than 1 mm in size [8]. A second-generation fluorescence laparoscope utilized LED lighting, which was an improvement of our previously described method of fluorescence laparoscopy and allowed for enhanced tumor detection without compromising background illumination [10]. With this advancement we were able to develop a method that is clinically translatable and has the potential to expand the role of diagnostic laparoscopy and surgical resection in cancer patients. There are now multiple commercially available near-infrared fluorescence imaging systems, the details of which are beyond the scope of this review.

Development of Tumor Selective Fluorescence Labeling

A variety of techniques have been developed to selectively label cancer cells with fluorescently tagged molecules that could ultimately be used to guide and improve surgical outcomes in cancer procedures. Carriers have been developed with increasing levels of sophistication that not only fluorescently label cancer cells for surgical resection, but also have the capacity to destroy remaining microscopic cancer. Here we will discuss a variety of preclinical developments. Table 1 summarizes the wide variety of developing applications of fluorescence-guided surgery in mouse models that will be discussed in further detail below.

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Monoclonal Antibodies Directed Against Cancer-Specific Proteins

Perhaps the most generalizable technique involves conjugating a monoclonal antibody directed against a known cancer biomarker to a fluorescent dye. In our laboratory, we developed an approach using monoclonal antibodies directed against biomarkers that are known to be associated with a variety of cancers—carbohydrate antigen19-9 (CA 19-9) and carcinoembryonic antigen (CEA). Monoclonal antibodies directed against these biomarkers were conjugated to a green fluorophore creating a delivery system that could target pancreatic and colorectal tumor cells and make them fluorescent. The purified antibody-dye conjugate was then injected intravenously into an orthotopic mouse model of human cancer 24 hours prior to whole-body imaging or fluorescence-guided laparoscopy. This widely applicable technique could be used for any cancer for which reliable biomarkers have been described. This strategy paves the way for personalized surgical medicine as more sophisticated techniques become available for finding individually complex targets and synthesizing targeted treatments.

Maawy *et al.* investigated the properties of commercially available near-infrared (NIR) dyes to delineate which dyes might be most appropriate for different applications in fluorescence-guided surgery. They compared fluorescence intensity of dye-antibody conjugates, tumor-to-background ratio, tumor depth, tumor size, photobleaching, hemoglobin quenching, and frozen section analysis of surgical margins [11]. The results indicated that longer-wavelength dyes had increased depth of penetration and had the highest sensitivity to detect the smallest tumor deposits. They also found that dyes with longer wavelengths had higher tumor-to-background ratios, were resistant to hemoglobin quenching, and were more specific. The lower wavelength dyes, however, were found to be more photostable [11].

Carbohydrate antigen 19-9 (CA 19-9) is a tumor marker that is found in up to 94% of pancreatic adenocarcinomas [12]. In 2008, we described for the first time conjugation of a monoclonal antibody specific for the tumor-associated antigen CA 19-9 with the AlexaFluor 488 green fluorophore and were able to demonstrate in vivo binding of the antibody-fluorophore conjugate to tumor tissue in a mouse model of human pancreatic cancer [13]. For the first time we were able to clearly visualized tumor deposits on the spleen, liver, and peritoneum that were otherwise invisible to the naked eye.

CEA is commonly associated with adenocarcinoma of the colon, but it is also often associated with pancreatic ductal adenocarcinoma [14,15]. Shortly after our success with fluorophore-labeled CA 19-9, Kaushal *et al.* demonstrated the successful use of fluorophore-labeled anti-carcinoembryonic antigen (anti-CEA) monoclonal antibody to fluorescently visualize tumor in nude mouse models of human pancreatic and colorectal cancer for FGS [16]. They showed fluorescence signals were detectable by 30 minutes after antibody-dye conjugate systemic administration, and that it remained present for 2 weeks.

Tran Cao *et al.* used fluorescence laparoscopy with fluorophore-conjugated antibodies (anti-CEA) to demonstrate significant increase in sensitivity to identify primary pancreatic tumors in an orthotopic model of pancreatic cancer compared to bright-light laparoscopy [8]. Fluorescence laparoscopy also increased the sensitivity and accuracy of staging laparoscopy

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to 96% compared to 40% for bright light laparoscopy. Fluorescence laparoscopy was sufficiently sensitive to detect metastatic tumor lesions that were less than 1 mm in size.

These studies set the groundwork for a revolutionary method for the surgical treatment of cancer. The next step was to look at how FGS could affect outcomes. In a proof of concept study, Metildi *et al.* compared traditional bright light surgery (BLS) to FGS for the resection of orthotopic mouse models of human pancreatic cancer. FGS resulted in a more complete resection (98.9% compared to 77.1% with BLS) and FGS resulted in significantly longer disease-free survival [17]. For the first time we saw that enhancing the distinction of tumor from surrounding normal tissue lead to improved outcomes. This was reiterated in a mouse model of human colorectal cancer where Metildi *et al.* fluorescently labeled orthotopic colorectal tumors and compared FGS to BLS for tumor resection. They discovered that small satellite tumors near the surgical margins were undetected in BLS but clearly visible with FGS [18]. The FGS group experienced significantly lower recurrence rates and had longer disease-free survival when compared to the BLS group. In another study Hiroshima *et al.* demonstrated FGS in combination with neoadjuvant chemotherapy (NAC) significantly decreased metastatic recurrence when compared to BLS alone, BLS + NAC, or FGS alone [19].

As discussed above, effective labeling of malignant tumors and metastatic focuses must maintain a clear distinction between the tumor and the surrounding tissue. This is called the tumor-background ratio (TBR) and it is affected by the concentration of dye in the target tissue versus the concentration that remains in the surrounding tissue [20]. Organs in the reticuloendothelial system display nonspecific uptake of antibody-dye conjugates, so identifying metastatic lesions in the liver, lung, lymph nodes and spleen has been a challenge [1,5,21]. Conjugation of molecules with polyethylene glycol (PEG), known as PEGylation, affects the biodistribution of the molecule and is widely used in pharmacology [22]. Maawy *et al.* demonstrated that PEGylation of two NIR antibody-dye conjugates increased the half-life, favorably altered biodistribution, and significantly increased the tumor to background contrast in liver and lung lesions [23]. PEGylated dyes had significantly lower accumulation in the liver when compared to non-PEGylated dyes, and this enabled high contrast imaging of metastatic tumor lesions of the liver [24].

Another cancer biomarker that shows promise is MUC1. MUC1 is a membrane bound glycoprotein that is overexpressed in approximately 90% of pancreatic cancer patients, which lends it to being a potential diagnostic biomarker and therapeutic target for the disease [25,26]. MUC1 is also frequently overexpressed in breast, ovarian, lung, and colon cancer, which makes an anti-MUC1 target applicable to multiple cancer processes [27,28]. Monoclonal antibodies to MUC1 were conjugated with DyLight 550 or 650 dyes in our laboratory and used to target orthotopically-implanted pancreatic cancer tumors grown from the BxPC3 or Panc1 human pancreatic cancer cell lines [29]. The study demonstrated that anti-MUC1 conjugated with a fluorophore could visualize pancreatic tumors both in vitro and in vivo in a nude mouse model.

There are a number of challenges associated with using antibody-dye conjugates, the most obvious of which involves the logistics of administration. Fluorescent visualization of

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targeted cancers is optimized when the antibody-dye conjugate is administered 24 hours prior to imaging, which is easily achieved in the lab. However, in practice optimal timing is more difficult to achieve and the fluorescent resolution before or after this point is dependent on the photostability of the conjugate, which varies considerably between dyes. Further, the shelf life of the antibody-dye conjugates has not been established. The next consideration relates to the administration of the antibody-dye conjugate in human patients and how these compounds might be tolerated. Given the variety of potential agents and applications, it would be prudent to establish a protocol of preclinical safety trials for the development of new fluorescent probes [30].

Adenovirus Vectors

Telomerase is a ribonucleoprotein enzyme that is involved with the replication of the ends of chromosomes, and its overexpression is thought to be responsible for the infinite replication potential of cancer cells [31]. OBP-301 and OBP-401 are conditionally replicating type 5 adenoviruses that are regulated by the human telomerase reverse transcriptase (hTERT) promoter [32]. hTERT is the catalytic subunit of telomerase that is highly active in cancer cells, but quiescent in most normal tissue [33]. Once infected, cancer cells acquire specific genes that the attenuated adenoviruses are engineered to carry.

OBP-401 is an attenuated adenovirus that contains the genetic coding sequence for the green fluorescent protein (GFP), and is selectively cytotoxic to cancer cells [34,35]. The virus is capable of entering most cells, but only replicates in the presence of active telomerase, which is characteristic of malignant but not normal cells [34]. In our lab, Yano *et al.* evaluated high dose intratumor (i.t.) injection of OBP-401 and were able to show that fluorescence guided surgery in combination with OBP-401 was curative for soft tissue sarcoma [36]. This technique has the dual ability to illuminate the gross tumor and destroy microscopic disease. The study showed that OBP-401 based FGS resulted in recurrence-free surgery, could enable minimally invasive, function-preserving surgery, and inhibited lung metastasis with high dose OBP-401.

In another experiment, Yano *et al.* were able to successfully label pancreatic cancer with RFP-expressing stroma using OBP-401 in a patient derived orthotopic xenograft (PDOX) mouse model [37]. They demonstrated an advantage of dual-color FGS over single color FGS or BLS for precise localization and complete resection of pancreatic cancer. In another study that demonstrates just how widely applicable OBP-401 can be, Yano *et al.* used OBP-401 based FGS on glioblastoma multiforme (GBM) in an orthotopic implantation mouse model [38]. OBP-401 was shown to label glioma cells in low doses, and in high doses provide enable less invasive, recurrence-free FGS by killing invading GBM cells. OBP-401 was able to label the cancer down to the single cell using the FV1000 confocal microscope. In another study Yano *et al.* successfully labeled and completely resected lung cancer tumors in an orthotopic mouse model for lung cancer [39].

Before this can translate into the clinic drug safety and toxicity profiles must be established. Phase I trials for the related OBP-301 (similar selectively cytotoxic adenovirus but without the GFP gene) were conducted which demonstrated that the therapy was well-tolerated [40].

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Herpes Simplex-1 Virus (HSV) NV1066

The herpes simplex virus type 1 mutant, NV1066, is a replication-competent virus that selectively infects and lyses tumor cells while sparing normal tissue and carries a transgene for EGFP [41]. Stiles *et al.* were able to show that after NV1066 treatment in vivo EGFP was expressed in cancer cells and tumor was visualized under fluorescence laparoscopy. NV1066 treatment in vitro and in vivo also resulted in cancer cell death [42]. Stanziale *et al.* showed NV1066 selectively infected and replicated within peritoneal cancer cells and resulted in both fluorescence labeling of tumor cells as well as cell death [43]. When used in combination with surgical resection, oncolytic HSV-1 may improve local control by targeting micrometastasis, ultimately improving long-term outcomes [41].

NV1066 was also used to target mouse models of lymphatic metastasis of human mesothelioma cancer cells. The virus was injected directly into primary tumors and was able to locate and replicate within metastatic lymph nodes that were then visualized with fluorescence imaging [44]. This technique also has the potential to be used as a highly sensitive diagnostic for cancer. Because of their ability to selectively label and detect even single cells, NV1066 has been used in ex-vivo cytological analysis of body fluids to detect cancer cells [45]. This technique is called fluorescence-assisted cytological testing (FACT), and by "highlighting" very rare cancer cells with GFP in a specimen of human body fluid it allows for their detection.

Activatable Cell-Penetrating Peptides

Another method of cancer cell fluorescence labeling takes advantage of the overexpression of cleaving enzymes known as matrix metalloproteinases (MMPs) that occurs in some cancers. Activatable cell-penetrating peptides contain negatively charged sequences that bind electrostatically to cells and when cleaved are able to enter the cell [46]. They can be covalently bonded to a wide variety of molecules including fluorescent dyes, which allow cancer cells to be labeled through a receptor-independent mechanism [47]. Nguyen *et al.* reported significantly improved outcomes when ACPP guidance was compared to bright light surgery in mouse models of human fibrosarcoma and melanoma [48]. Metildi *et al.* demonstrated that MMP-2 and MMP-9-cleavable ratiometric activatable cell-penetrating peptides (RACPPs) conjugated to Cy5 and Cy7 fluorophores could effectively label pancreatic cancer in an orthotopic mouse model, and that FGS using this method reduced metastasis and recurrence [49].

Anti-Calreticulin (Anti-CRT) Conjugated to Gold Quantum Dots (QDs)

Gold quantum dots (AuQDs) have been applied to FGS. Gold quantum dots are semiconductor nanocrystals that can be precisely engineered to a desired fluorescence with high biostability [50]. Compared to organic dyes and fluorescent proteins, QDs are brighter, more stable and have a more narrow emission spectrum [51]. They can be conjugated to antibodies and loaded with therapeutic agents. Giorgakis et. al. demonstrated that another biomarker, calreticulin (CRT) is overexpressed in solid pancreatic lesions (premalignant, malignant, adenocarcinomatous, and neuroendocrine tumors), and that polyclonal antibodies against CRT could be conjugated to fluorescent gold quantum dots [50]. However,

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nanoparticle technology is an entirely new drug delivery system, and untoward side effects or toxicities remain largely unknown [52].

Photoimmunotherapy

Another exciting advancement with potential for FGS is a technique termed photoimmunotherapy (PIT). As with other approaches describe above, PIT has the dual ability to localize tumor and assist with surgical navigation as well as selectively eliminate cancer cells. PIT uses a monoclonal antibody specific for known cancer biomarkers that are conjugated to a photosensitizer pthalocyanine dye IR700 [53]. The complex becomes cytotoxic upon exposure to near infrared light [54]. These cytotoxic effects are observed only when the monoclonal antibody-IR700 (mAb-IR700) complex was bound to the cell membrane; no phototoxicity was observed when mAb-IR700 was not bound [55]. In a proof of principle study, Maawy *et al.* demonstrated that PIT, using anti-CEA-IR700, causes extensive cell death in vitro when bound to pancreatic cancer cells known to express CEA, and that a one-time treatment of PIT results in a significant reduction in tumor size and weight in an orthotopic mouse model of human pancreatic cancer [56]. In an orthotopic mouse model of BxPC3 pancreatic cancer, BLS in combination with PIT reduced local recurrence to 1/7 mice from 7/7 mice when compared to BLS alone and metastatic recurrence to 2/7 compared to 6/7 with BLS only [57]. Similarly, in a pancreatic cancer patient-derived orthotopic xenograft (PDOX) nude mouse model local recurrence was decreased from 85.7% in BLS mice to 28.6% when BLS was combined with PIT [58]. Future studies could replace BLS with FGS, which may lower local recurrence even further. Additionally, serial PIT treatments could be studied to establish a more effective regimen.

γ-Glu-Hydroxymethyl Rhodamine Green (γ-Glu-HMRG)

Urano *et al.* developed a simple and effective approach to rapid intraoperative labeling of cancer cells taking advantage of a cell surface enzyme that is overexpressed in many cancers but not found in high amounts in most normal tissue [59]. γ-glutamyltranspeptidase (GGT) is a membrane-bound aminopeptidase that regulates glutathione homeostasis and is thought to promote tumor progression, invasion, and drug resistance by potentially altering intracellular redox metabolism [60,61]. γ-Glu-HMRG is activated into a fluorescent probe in a one-step enzymatic reaction in the presence of GGT.

In a mouse model of human ovarian cancer Urano *et al.* sprayed the abdominal cavity with the γ-Glu-HMRG probe and demonstrated that small tumor nodules were visualized in as few as 10 seconds after administration and remained for one hour. Metastatic implants as small as 1 mm were removed under fluorescence-guided laparoscopy [59]. Mitsunaga *et al.* used the γ-Glu-HMRG during colonoscopy to differentiate long-term colitis from early colitis-associated cancer (CAC). They were able to visualize cancer and dysplasia 5 to 30 minutes after topical administration of the γ-Glu-HMRG with a fluorescent signal that was in general 10 times high than background colitis [62].

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Current Clinical Use

The era of fluorescence-guided imaging for surgical oncology has arrived. New applications continue to be described as even more are developed. Novel, ultra-sensitive techniques for the localization and destruction of cancer are emerging from pre-clinical research into the operating room. Fluorescence imaging enhances cancer surgery navigation and offers higher sensitivity when compared to preoperative imaging, visual inspection and palpation during surgery [4]. Here we will focus on the current use of fluorescence imaging in surgical oncology. Table 2 summarizes the current clinical uses of FGS.

Indocyanine Green (ICG)

Indocyanine green (ICG) is an FDA-approved, non-toxic dye that emits a fluorescent signal when excited by near-infrared light in the 700-900 nm wavelength spectrum [63]. It has been used clinically for over 50 years and has a high safety index (1:300,000 adverse reaction rate with a maximum recommended dose of up to 2 mg/kg) [64,65]. ICG is taken up by the hepatocytes and excreted through the bile, which lends this system to real-time imaging of the hepatobiliary system [66]. Several commercially available systems exist for open, laparoscopic, and robotic surgeries. ICG is a non-selective fluorophore that can be exploited clinically for its ability to display relative hyper/hypoperfusion between tumor and normal tissue, biliary anatomy, washout differences between tumor and adjacent parenchyma, objectively assessing micro perfusion of anastomotic sites, and for lymph node mapping.

Tumor Imaging in Hepatobiliary Disease—As mentioned previously, ICG is excreted exclusively by the liver through the bile system [67]. As a result liver neoplasms cause characteristic aberrations in bile excretion that are visualized with near-infrared (NIR) fluorescent imaging systems [68]. Due to cellular dysfunction observed in hepatocellular carcinoma and colorectal cancer liver metastasis, the hepatocytes are able to take up the ICG dye, but are unable to excrete it into the biliary system [69]. Moderately to well-differentiated HCC tends to fluoresce brightly throughout the tumor, while poorly differentiated cancer tend to be rim-enhancing, a phenomenon thought to be due to compression against normal bile ducts [69]. Liver neoplasms can be clearly visualized with specialized NIR imaging systems after ICG is cleared from the normal-functioning liver parenchyma. Use of these fluorescence imaging systems at the time of surgery may be more sensitive than current imaging modalities. Employing intraoperative ICG imaging, Uchiyama *et al.* identified several additional lesions that were not seen on preoperative CT or MRI and that these findings altered the operative strategy [70]. Since ICG is an FDA-approved drug and imaging systems for laparoscopic, robotic, and open surgery are commercially available, this technique may be widely employed in hepatobiliary surgery in the near future.

Fluorescence Cholangiography—Perhaps the application with the most potential for widespread adoption is ICG fluorescence cholangiography. Laparoscopic cholecystectomy is the most common surgical case performed in the United States, with over 750,000 cases performed annually [71]. Since ICG is taken up by the hepatocytes and eliminated through the bile near-infrared imaging can be employed to visualize extrahepatic biliary anatomy

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during laparoscopic cholecystectomy prior to dissection. Unlike conventional cholangiography, fluorescence cholangiography can be performed in real time with one of several commercially available specialized laparoscopes without disrupting workflow with bulky equipment. Transition between bright light and fluorescence mode is quick and easy, allowing for frequent reorientation and confirmation of critical biliary structures.

Ishizawa *et al.* first described fluorescent cholangiography in 2010 in a series of 52 patients undergoing laparoscopic cholecystectomy [72]. Identification of the cystic duct-common hepatic duct junction during dissection of Calot's triangle was achieved in real time using a specialized laparoscope in 50 of 52 patients. The technique does not require specialized training, it is easy to perform, and is done without the use of ionizing radiation. Preoperative injection of ICG approximately one hour prior to surgery allows for complete uptake by the hepatocytes and excretion into the biliary system. Fluorescence cholangiography may replace traditional intraoperative cholangiogram for elective laparoscopic cholecystectomy. Further studies will delineate how fluorescence cholangiography may affect common bile duct injury.

Sentinel Lymph Node Mapping—The standard approach for localizing sentinel lymph nodes involves the dual use of a gamma-emitting radiotracer and a blue dye, a technique that requires a nuclear medicine physician in addition to an experienced surgeon. NIR fluorescence imaging can visualize the flow of ICG in real time and has been studied as an adjunct or replacement of currently used methods [73]. In a prospective study of 301 patients with breast cancer, Samorani *et al.* observed a very high concordance when comparing ICG to the radioisotope technetium suggesting that ICG may be an acceptable alternative [74]. Since ICG can visualize lymphatic channels in real time, this technique may prove to be useful for lymph node mapping in a wide variety of cancers.

Assessment of Microperfusion and Angiography Mapping—Anastomotic leaks contribute significant morbidity to many cancer surgeries that require major surgical resections. Among the known risk factors that contribute to anastomotic leaks is poor perfusion to the adjacent tissue. NIR fluorescence imaging has a long history of use by plastic surgeons to assess microperfusion in flaps and tissue transfers [75]. Zehetner *et al.* utilized NIR fluorescence imaging in a series of patients undergoing esophagectomy to identify the right gastroepiploic artery and to evaluate the perfusion of the tubularized gastric graft [76]. They identified differences in graft perfusion between patients and found that when the tip of the graft was brightly illuminated, anastomotic leak rates were trivial. This technique has also been used in colorectal surgery to assess anastomotic perfusion and in some cases has led to revision of the anastomosis [77].

5-Aminolevulinic Acid

Stummer *et al.* labeled tumor tissue in patients with malignant glioma. 5-aminolevulinic acid (5-ALA) is a metabolite precursor of hemoglobin without fluorescence that can be taken orally to label malignant glioma cancer cells [78]. Once inside malignant glioma cells, 5-ALA promotes synthesis and accumulation of fluorescent porphyrins, which allows the cancer to be visualized by a modified neurosurgical dissection microscope [79,80]. In a

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mouse model of human U87 glioma, it was demonstrated that almost all tumor could be removed using FGS without damage to the surrounding brain tissue [81]. The results in human trials have been equally striking.

In one study of 322 patients undergoing surgical resection for malignant glioma, subjects were given 5-ALA orally before undergoing bright light or fluorescence-guided neurosurgery. In the FGS group complete tumor resection was accomplished in 65% of the 139 patients, while only 36% of the 131 patients in the bright light surgery group had complete resection. Additionally, patients in the fluorescence-guided neurosurgical group had significantly improved 6-month progression-free survival rates (41% compared to 21% in the bright light group) [82]. This method effectively enhances the distinction between normal and abnormal tissue in malignant glioma, leading to impressive improvements in outcomes. However, it may not have a role in cancer surgery outside of malignant glioma.

Folate Conjugated to Fluorescein Isothiocyanate for Surgical Treatment of Ovarian Cancer

Folate receptor-α (FR-α) is often overexpressed in epithelial ovarian cancers [83]. Van Dam, *et al.* were able to conjugate folate to fluorescein isothiocyanate (folate-FITC) to selectively label ovarian cancer cells in 10 ovarian cancer patients who were undergoing abdominal surgery. Folate-FITC excites at a wavelength of 495 nm and emits at 520 nm [11,84]. A real-time multispectral intraoperative fluorescence imaging system was used to visualize and resect tumor deposits less than one millimeter [85]. In patients with peritoneal carcinomatosis, significantly more tumor deposits were identified when using fluorescence imaging compared to BLS. FR-α targeting is limited only to those tumors that bear overexpression of the folate receptor.

Anti-EGFR (Cetuximab) Conjugated to IRDye800 for FGS of Head and Neck Cancer

Rosenthal et. al. performed the first in human fluorophore-conjugated-antibody mediated FGS for head and neck cancer. Anti-EGFR (Cetuximab, ImClone LLC, Eli Lilly and Company) antibody was used since over 90% of head and neck tumors overexpress EGFR [86,87]. IRDye800 was selected due to a known lack of toxicity in previous rodent and non-human primates studies as well as existing commercially availability of near infrared imaging systems for dyes in that wavelength [88,89]. Fluorescence corresponded to tumor histology [87].

Expert commentary

The ability of the surgeon to accurately visualize tumor margins and identify metastases is necessary for the success of any cancer operation. Fluorescence optical imaging, because of its high sensitivity, low cost, portability, and real-time capabilities has great potential to improve surgical outcomes. Several different applications of fluorescence technology have already been used clinically. Surgeons are now routinely using ICG for imaging bile ducts during laparoscopic cholecystectomy and for assessment of bowel perfusion during colorectal and esophageal surgery. There have been several recent clinical studies of FGS for ovarian and lung cancers using folate conjugated FITC and head and neck cancers using cetuximab-conjugated to an 800 nm dye.

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A number of limitations of the aforementioned advances exist that must be addressed. First, we must improve our ability to target cancer cells. Cancer is a largely heterogeneous disease process and a gene expression profile of one cancer can have an enormous variation when compared to another. Hiroshima *et al* explored the use of multiple antibodies, anti-CA 19-9 and anti-CEA, directed toward the same types of tumor [19]. The heterogeneity of tumors demands a multifocal approach when it comes to labeling if we are to achieve a maximal sensitivity. As our targets increase in selectivity we will likely see a transition to combination 'cocktail' treatments. The ability to characterize a patient's cancer and synthesize targeted treatment will allow FGS to enter the realm of "personalized surgical medicine." Second, we must establish the safety profiles of many of these exciting new approaches. New delivery systems for fluorescent agents and therapeutics can now be packaged within nanoparticles to precisely target cancer cells. Unanticipated toxicity of this new class of diagnostic and therapeutic agents remains unclear. Similarly, the safety profile of the adenovirus vector approach with a seemingly ubiquitous application is largely unknown. Preclinical safety protocols must be developed to streamline the process and overcome the enormous financial burden associated with new pharmaceuticals.

Five-year view

The ability to define cancer markers and engineer targeted therapies continues to rapidly evolve. As techniques continue to increase in complexity we will be moving ever closer toward the concept of "personalized surgical therapy." It is possible that future FGS will be personally designed for a patient's specific disease process. Our "one size fits all" solutions will be replaced with "precision medicine," tailoring treatments to an individual's unique disease process. Fluorescent imaging systems will likely become commonplace in the operating rooms on standard laparoscopic imaging systems or as an adjunct for open surgical cases. In conclusion, there are many approaches to FGS using antibodies, small molecules, and viral labelling. More tumor-specific labels will be discovered shortly. Every cancer surgery should be fluorescence-guided in the near future.

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Key Issues

- Curative cancer surgery is dependent on the removal of all tumor and metastatic cancer cells.
- Preoperative imaging, intraoperative inspection and palpation, and pathological margin confirmation aid the surgeon, but these methods are lacking in sensitivity and can be highly subjective.
- Techniques in fluorescence-guided surgery (FGS) are emerging that selectively illuminate cancer cells, enhancing the distinction between tumor and surrounding tissues as never before with potential single-cell sensitivity.
- FGS enhances tumor detection, surgical navigation, margin confirmation, and, in some cases, can be combined with therapeutic techniques to eliminate microscopic disease.
- Fluorophore-conjugated antibodies to tumor-specific antigens have been used in preclinical and clinical FGS.
- Viral vectors such as adenovirus and herpes virus can be used to deliver fluorescent proteins to cancer cells.
- Activatable cell-penetrating peptides have been engineered to highlight tumors based on enzymatic cleavage of peptidases.
- Indocyanine green (ICG) is being used clinically to aid in defining liver tumor margins, tissue perfusion, and biliary anatomy.
- Fluorescent imaging systems will likely become commonplace in the operating rooms on standard laparoscopic imaging systems or as an adjunct for open surgical cases.

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Table 1

Preclinical techniques for in vivo labeling of cancer cells with fluorescence

Technique	Tumor Type	Fluorescence Type	References
Fluorophore-conjugated antibodies	Pancreas, colon	Alexa 488 or 550, various other	[10,13,16,90]
Activatable cell penetrating peptides	Melanoma, sarcoma	Cy5, Cy7	[48]
Telomerase-dependent Adenovirus (OBP-401)	Gastric, pancreas, glioma, sarcoma, lung, colon	GFP	[34,91-93]
Oncolytic herpes simplex-1 virus	Esophageal, mesothelioma	GFP	[42-45,94]
Quantum dots	Pancreas	Quantum dots	[51,52,95]
Photoimmunotherapy	Pancreas	IR700	[53,56-58]
γ-Glu-HMRG	Ovarian	Rhodamine green	[59,62]

gGlu-HMRG = γ-Glutamyl hydroxymethyl rhodamine green; GFP = green fluorescent protein.

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Table 2

Currently used techniques for in vivo labeling of cancer cells with fluorescence

Technique	Tumor Type	Fluorescence Type	References
Labelled folate	Ovarian	FITC	[85]
5-aminolevulinic acid	Malignant glioma	Porphyrin	[78,80,82]
Indocyanine green (ICG)	Hepatocellular carcinoma	ICG	[69]
Methylene Blue (MB)	insulinomas	MB	[96]

FITC = fluorescein isothiocyanate