Intraoperative Imaging-Guided Cancer Surgery: From Current Fluorescence Molecular Imaging Methods to Future Multi-Modality Imaging Technology

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Abstract

Cancer is a major threat to human health. Diagnosis and treatment using precision medicine is expected to be an effective method for preventing the initiation and progression of cancer. Although anatomical and functional imaging techniques such as radiography, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) have played an important role for accurate preoperative diagnostics, for the most part these techniques cannot be applied intraoperatively. Optical molecular imaging is a promising technique that provides a high degree of sensitivity and specificity in tumor margin detection. Furthermore, existing clinical applications have proven that optical molecular imaging is a powerful intraoperative tool for guiding surgeons performing precision procedures, thus enabling radical resection and improved survival rates. However, detection depth limitation exists in optical molecular imaging methods and further breakthroughs from optical to multi-modality intraoperative imaging methods are needed to develop more extensive and comprehensive intraoperative applications. Here, we review the current intraoperative optical molecular imaging technologies, focusing on contrast agents and surgical navigation systems, and then discuss the future prospects of multi-modality imaging technology for intraoperative imaging-guided cancer surgery.

Key words: Optical molecular imaging; Intraoperative imaging-guided cancer surgery; Near-infrared fluorescence; Multi-modality Imaging; Indocyanine green.

Introduction

Presently, nearly 13 million new cancer cases and 7.6 million cancer deaths occur worldwide each year [1]. From 1971 to 2011, the National Cancer Institute (NCI) spent about $90 billion on research, treatment, and prevention of cancer and is approaching a 5-year doubling of its budget [2-4]. The past decade has witnessed the rapid growth and technological advancement of imaging techniques; many of which have been applied for preoperative tumor diagnosis, most notably: radiography, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission computed tomography (SPECT). However, these techniques are for the most part not applicable to intraoperative tumor surgery, for which palpation and visual inspection remain the predomi-
Fluorescence molecular imaging (FMI) has been established as a powerful tool for guiding precise intraoperative positioning [6-9]. This technique can be described as a fluorescent labeling method that uses an imaging system to help surgeons distinguish between normal and malignant tissues labeled through the injection of a fluorescent detection agent. Over the past several years, this technology has improved the ability to surgically treat liver metastases [8], breast cancer [9-12], ovarian cancer [7], melanoma [13, 14], vulvar cancer [15, 16] and cervical cancer [17, 18].

Defining a way to objectively assess tumor margins during surgery plays a key role in diagnostic precision [19]. Traditionally, surgeons determine the tumor resection margin based on their experience and submit the specimen for histological evaluation. If the margin proves positive upon pathology, additional removal will be executed. Cytoreductive surgery followed by combination chemotherapy is also considered an effective treatment. The degree of cytoreduction predicts the tumor recurrence and survival, even if the residual tumor diameter is less than 1 cm after therapy [6, 7]. Remarkably, current studies suggest that FMI technology can assist surgeons in resecting micro-cancer tissues down to the submillimeter size, thus improving patient outcome [20-22].

With the aid of FMI technology, the prognosis of patients will as a result improve. For example, a study of breast cancer survivors at risk for breast cancer-related lymphedema found that early accurate treatment results in a better outcome and even complete resolution of lymphedema-associated complications [23]. Suzuki et al. used the FMI method with orally administered 5-aminolevulinic acid (5-ALA) to improve the prognosis of patients with glioma [24]; Loja et al. used Alexa-647 labeled pHLIP (pH responsive peptide conjugated with Alexa Fluor (R) 647) to detect alterations in extracellular pH found in head and neck squamous cell carcinoma (HNSCC) in order to assess tumor margins during surgery, thus promoting better detection and prognosis of this cancer [25].

The most important components of FMI technology are the imaging contrast and surgical navigation system. Their further development will provide surgeons with intraoperative image guidance and information about residual tumors on follow-up. For clinical applications, the Food and Drug Administration (FDA) needs to approve fluorescence imaging contrast agents. The fluorescent dye indocyanine green (ICG) obtained approval and has been applied to sentinel lymph node (SLN) mapping [10, 12, 17, 26] and hepatic micrometastases detection [27].

However, ICG lacks precise targeting properties. To enable more selective tumor detection, fluorescent dyes can be chemically conjugated with targeting moieties such as peptides, antibodies, or sugars, which are systemically metabolized and accumulate in lesion sites [28]. These fluorescent dyes, which are still in a preclinical stage, show potential as markers of cancer cells, tumor angiogenesis, and tumor microenvironments, but there are likely still a long way from FDA approval.

After injection of fluorescent dyes, a surgical navigation system is required to indirectly activate these dyes by supplying the tissues with near-infrared (NIR) light. Video rate images are provided by the system for accurate surgical guidance after fast image registration processing by a computer [29]. These techniques are therefore useful for providing surgeons with precise tumor detection in real-time during surgery.

FMI technology has proven to be a promising method for intraoperative tumor detection in clinical applications, although it is limited to the body’s periphery. Therefore, other modalities have been introduced into image-guided surgery for more complete visualization of tumors. Kircher et al. reported a triple-modality MRI-photoacoustic-Raman method that helped to more accurately delineate the margins of brain tumors in living mice both preoperatively and intraoperatively [30]. This technique demonstrated the feasibility and advantages of multi-modal image-guided methods for precise characterization of the tumor margin during surgery.

To provide a better understanding of FMI technology, this review will focus on the innovative fields of instrumentation in clinical applications of FMI technology during cancer surgery. Owing to the current limitations of FMI technology, we will also describe methods for using multi-modalities and the potential long-term translational benefit for patients.

### Surgical Navigation Systems

Intraoperative FMI technology relies on the availability of intraoperative imaging system and an imaging contrast agent to visualize the carcinoma in situ and metastatic lesions during surgery. Based on the tissue penetration depth, a high level of signal-to-background ratio (SBR) is required. The NIR range is between 700 and 900 nm, at which light absorption and scattering are relatively low [31]. In recent years, the concept of using NIR fluorescence imaging has now been demonstrated experimentally, a crucial step towards its application in intraoperative image-guided surgery.

As NIR light cannot be seen directly with the naked eye, many academic and industrial groups have been devoted to the development of various...
imaging systems for intraoperative NIR FMI over the past few years [10, 29, 32-43]. Now, most of these systems have been applied to clinically and have made progress in assisting intraoperative tumor surgery. Besides attaining FDA approval, there are some important challenges for optimization of these systems for clinical use, including: real-time white light and fluorescence visualization, optimized NIR light-sources for sufficient fluorescence excitation, and convenience in clinical translation.

Several existing intraoperative FMI systems are available for clinical studies (Table 1). Existing systems can be classified into three categories as portable, functional and endoscopic and laparoscopic intraoperative FMI systems. Most of these systems have already been applied to clinical diagnosis and treatment. Furthermore, different types of systems have different performance focus. They have played an important role in operation convenience, improving image assessment and increasing detection depth. Now there are three systems have passed FDA approval. The Hamamatsu’s (www.hamamatsu.com) Photodynamic Eye (PDE™), Artemis™ (www.o2view.com) and Novadaq SPY™ system (Novadaq Technologies Inc., Toronto, Canada) have already been applied in surgeries for breast cancer [9, 44], liver metastases [43] and bypassing graft surgery [46-51].

**Portable intraoperative FMI systems**

These surgical navigation systems are intended to assist in surgery and portable systems satisfy important criteria for convenient operation. An effective system for intraoperative imaging needs to be able to readily facilitate the operation. PDE™ is a hand-held imaging system that emits annular NIR light and detects it through the in vivo uptake of fluorescent 2D images. The handheld capability and performance of the Fluoptics (www.fluoptics.com) Fluobeam® are similar to PDE, and it is now being used in clinical trials aiming to demonstrate the ability of FMI during and after an operation. PDE and Fluobeam possess the advantage of being compact and convenient for real-time fluorescence imaging. With the benefit of intraoperative visible images, Artemis™ simultaneously shows the color image and the fluorescent overlay, which provides excellent utility for nerve surgery [52, 53].

In the field of breast oncology, the SPY system has recently been applied to monitor skin perfusion in nipple-sparing mastectomies with ICG. This method can be seen as a useful adjunctive tool with potential to enable direct placement of mastectomy incisions and minimize ischemic complications [54]. Recently, some other interesting intraoperative imaging systems have been developed for clinical applications. For example, in the goggle system developed by the Department of Radiology at Washington University, the surgical navigation system is projected on wearable glasses, which frees the surgeon’s hands so that an operation can be performed perform with more flexibility [43].

**Table 1. Parameter comparison of image-guided systems.**

| No. | Imaging systems | Manufacturer                          | Main application                  | Excitation wavelength (nm) | Field of view (mm) | Resolution | Display Refresh (Hz) | Dy-namic range (bits) | Working distance (mm) | Color video source | Clinical | Status        | Reference                  |
|-----|-----------------|--------------------------------------|-----------------------------------|----------------------------|--------------------|------------|---------------------|----------------------|----------------------|---------------------|----------|---------------|--------------------------|
| 1   | SPY             | Novadaq Technologies, Mississauga, Canada | Intraoperative Fluorescence Imaging | 820                        | 190*127            | Not specified | 30                  | 8                    | 300                  | No                  | Laser               | FDA approved             | www.novadaq.com          |
| 2   | Artemis         | O2view, Marken, The Netherlands       | Stereoscopic Fluorescence Imaging | 400-1000                   | 22.5*22.5          | 5-60        | 14                  | ≥50                  | 200                  | No                  | LED                 | FDA approved             | www.o2view.com           |
| 3   | Photodynamic Eye | Fluoptics, Grenoble, France           | Handheld Fluorescence Imaging     | 760                        | 100*67            | Not specified | 8                   | 200                  | No                  | LED                 | FDA approved             | www.hamamatsu.com        |
| 4   | Fluobeam        | Fluoptics, Grenoble, France           | Handheld Fluorescence Imaging     | 690 or 780                | 128*94            | 30          | 12                  | 150                  | No                  | Laser               | Clinical trial          | www.fluoptics.com        |
| 5   | SurgOptix       | SurgOptix, Redwood Shores, USA        | Intraoperative Fluorescence Imaging | 520                        | 115*93            | 12          | 16                  | 210                  | Yes                 | Laser               | Clinical trial          | www.surgoptix.com        |
| 6   | FLARE           | Frangioni Laboratory, Boston, USA     | Intraoperative Fluorescence Imaging | 670 or 760               | 150*113            | 15          | 12                  | 450                  | Yes                 | LED                 | Clinical trial          | www.centerformonitoring.org | www.3dmed.net          |
| 7   | GXM Navigator   | Institute of Automation, Beijing, China | Intraoperative Fluorescence Imaging | 760nm                     | 250*250           | 17          | 16                  | >300                 | Yes                 | LED                 | Clinical trial          | http://www.thno.org      |
Functional intraoperative FMI systems

These functional systems have performance advantages in image capture and processing. The FLARE™ imaging systems created in the Frangioni Laboratory (www.frangionilab.org) uses three cameras to simultaneously collect images from two different NIR channels and one visible channel. FLARE and mini FLARE systems are being tested in clinical trials, and have been applied to several forms of cancer surgery, most notably for intraoperative SLN mapping [8, 10, 12, 55, 56]. The multispectral FMI system from Technische Universität München & Helmholtz Zentrum (http://www.helmholtz-muenchen.de) produced in collaboration with SurgOptix (SurgOptix Inc., Redwood Shores, CA, USA) has the advantage of being able to correct for the attenuation of the excitation of light and also can be applied to clinical cancer research [7, 15, 17, 57-59]. These two imaging systems perform well in multi-spectral imaging and improve image quality.

Another surgical navigation system is the GXMI Navigator developed in our Key Laboratory of Molecular Imaging at the Chinese Academy of Sciences (http://www.3dmed.net/). This device retains the advantage of convenient operation, while also improving the quality of imaging results [60]. Similar to the shadowless lamp, the system uses feature points to ensure rapid and precise imaging through the fusion of two cameras and has been applied in SLN mapping of patients in early stages of breast cancer. The systems are all are shown in figure 1.

Endoscopic and laparoscopic FMI systems

In recent years, endoscopic and laparoscopic systems have been coupled with FMI technology to solve the detection depth problem. These endoscopic and laparoscopic systems have been successfully applied in cancer surgery and have assisted in minimally invasive cancer therapy [61]. However, one cannot palpate tumors or handle the tumor-riddled organs via endoscopic or robotic surgery. It will be great help for surgeons to dissect tumors with guidance from high resolutions SBR images. Yokoyama et al. used the ICG fluorescence method to aid in endoscopic surgery for head and neck cancer. This technique produced fluorescent images with a significant contrast between tumor and normal tissues [62].

Wide application of FMI technology in endoscopic and laparoscopic surgery faces the challenging problem of how to adjust the endoscopic optical path to simultaneously achieve visible and NIR fluorescent images. When there is an established component between the endoscope and camera, it is difficult in many existing endoscopic applications to change the regular workflow and integrate the FMI technology.

Several studies have however demonstrated successful integration of fluorescence imaging capabilities into experimental endoscopic and laparoscopic systems. Oh et al. presented a wide-field multi-channel fluorescence endoscopic system for early detection and treatment of colon cancer with matrix metalloproteinases (MMP) conjugated with quantum dots [63]. Matsui et al. used a custom NIR fluorescence laparoscopy system to identify the extrahepatic bile ducts in Yorkshire pigs. The results demonstrated...
good sensitivity for the identification of extrahepatic bile ducts and their functional status [64]. This laparoscopy system was also successfully applied for the fluorescence-guided identification of the ureters using methylene blue in pig experiments [65].

Based on these previous achievements, Venu-gopal et al. built a prism-based dual-channel endoscopic imaging system compatible with two light sources in color and one in the NIR range with a 2-CCD camera. This system demonstrated two key advantage: 1) the imaging camera was compact enough to be operated easily by one hand during surgery and 2) the optical channels allowed for accurate registration for real-time image processing [66]. Another laparoscopy system similar to the dual-channel endoscopic system has also been described. Glatz et al. presented a video-rate color and NIR fluorescence laparoscopy system for the identification of colorectal tumor margins [67]. Taken together these advances indicate that endoscopic and laparoscopic systems with FMI technology are rapidly approaching clinical translation and will be available for use in patients in the near future [68].

**Multi-modal intraoperative surgical navigation system**

Intraoperative surgical techniques require development of dedicated intraoperative image-guided systems. Important progress has been achieved over the past few years in both fields; however, multi-modal imaging methods and systems are still needed for clinical translation of these technologies. Currently, several fluorescence-imaging systems are already commercially available. However, image-guided intraoperative systems supplying three-dimensional precision tumor detection are still unavailable [7, 12, 60].

Detection depth is the main limitation of the FMI method for some clinical applications. However, if this problem were overcome, this method would allow for intraoperative tumor margin detection, which would be clinically meaningful to both surgeons and patients. Questions remain regarding how to solve the depth limitation challenge, which is essential in enhancing the value of FMI technique and multi-modal methods. Ale et al. examined the *in vivo* performance of a camera-based hybrid fluorescence molecular tomography (FMT) system for 360 degree imaging combined with X-ray computed tomography (XCT). This multi-modal method can provide concurrent anatomical and functional information. These findings indicate that FMI combined with the XCT method can increase the detection depth and may provide a substantial advance for current XCT applications [69].

Si et al. combined a single-cycle pulsed ultrasound modulation with digital optical phase conjugation. This multi-modal method has adequate optical power to focus light within high-scattering media for not only fluorescence imaging, but also numerous linear and nonlinear spectroscopy measurements. Xie et al. designed PET/NIR/MRI triple modality functional nanoparticles to achieve personalized tumor treatment. The results demonstrated that a tumor can be visualized *in vivo* in the U87MG xenograft mouse model by this triple modality [70]. As the diffuse gliomas are not always detected by contrast-enhancement MRI, Ewelt et al. introduced a method performed in 30 patients using ¹⁸F-FET and Gadolinium-enhanced MRI for pre-operative diagnosis and 5-aminolevulinic derived tumor fluorescence for intraoperative image-guided malignant glioma dissection. The results showed 70.5% sensitivity and 92.3% specificity using the multi-modal method, which supplied valuable pre-diagnostic imaging information and assisted in fluorescence-guided tumor resection [71].

In summary, the multi-modal imaging systems and technology can compensate the limit of detection depth in fluorescence imaging by imaging processing method and set the standard for many important applications in biological research and clinical trials [72].

**Clinical Applications**

**Clinical applications using ICG**

Although many NIR fluorescent molecules have been developed for tumor targeting, it will take time for their translation into clinical applications. The FDA approved molecule ICG can be used in ocular, cardiocirculatory, and liver function diagnostics. Recent reports focusing on intraoperative SLN mapping and HCC detection have further extended its clinical application.

Tumor metastasis usually occurs through the lymphatic system and the SLN is typically the first anatomical location of tumor metastasis. Currently, most researchers performing SLN mapping use a visible blue dye (such as isosulfan blue, patent blue, or methylene blue), a radioactive tracer (such as ⁹⁹mTc or sulfur colloid), or a combination of the two. The blue dye is widely used for its low price and ease of use. However, the light emitted by the dye cannot pass through the skin and adipose tissue if the dose is not properly controlled. Further, some side effects such as tissue necrosis may result from the dye. In fact, the rate of successful tumor identification using a single dye is not satisfactory [73]. The surgeon may instead employ radioactive detection methods in which a radiation counter is used to detect and locate the SLN on
the surface of the tissue with the radioactive element $^{99m}$Tc. The two methods for intraoperative detection and localization are often combined to improve the detection rate of SLN [73, 74]. Although this combination method improves detection results, side effects and radioactive hazards still remain problematic.

FMI using ICG provides new opportunities to improve the SLN procedure [75]. The application of ICG shows a high SBR, which helps the surgeons locate the SLN before excision through real-time observation. As ICG is clinically available, many intraoperative imaging systems have focused on SLN cases. NIR fluorescence image-guided SLN mapping has been extensively used in oropharyngeal [56, 76], breast [10, 77-84], melanoma [13, 85-90], lung [91-98], esophageal [99], gastric [100-113], cervical [18, 114-117], colorectal [42, 55, 118-121], endometrial [122-124] and vulvar cancer cases [15, 16, 125]. The results have demonstrated the feasibility of intraoperative SLN mapping using FMI technology.

As blue dye can be visualized with the naked eye and has already been widely used in SLN mapping studies, the combined use of blue dye and ICG has been assessed. Sugie et al. found that a high rate of SLN detection was achieved using the ICG fluorescence method [9]. Since the optical penetration depth (<10 mm) limits the visualization of deep tissues, Verbeek et al. used blue dye, ICG and radionuclides in combination, which increased the detection accuracy rate (rising to 78 % for blue dye, 100 % for NIR fluorescence, and 88 % for radioactivity) [126].

Although blue dyes, ICG, and radionuclides can effectively trace the location of SLNs, they also travel from the injection site to higher tier nodes. To prevent migration, dyes are modified to inhibit migration; radionuclides are commonly conjugated to a colloid to increase the hydrodynamic diameter or to a ligand to increase retention [127]. For fluorescent dyes, the choice of conjugant can affect the optical properties, as ICG cannot be conjugated covalently to sulfur colloids without altering its chemical structure [128]. The performance of targeting tracers should be further evaluated in direct comparison to existing agents for clinical use.

As the FDA approval of ICG has played an important role in clinical SLN mapping, some recent studies have reported on the detection of HCC using intraoperative FMI technology [45, 129]. The strongest fluorescent signals in patients were found if ICG was given several days before surgery as a routine preoperative liver function test [40, 129-133]. These reports offer new opportunities for intraoperative pancreatic tumor visualization.

With the advent of FMI techniques and systems, intraoperative liver cancer and metastases resection is becoming a simple, low-risk, and highly sensitive procedure. Ishizawa et al. used ICG at the dose of 0.5 mg/kg for routine liver function testing within 2 weeks of surgery to achieve highly sensitive identification of HCC through the visualization of noncancerous liver parenchyma around the tumor. The results showed that intraoperative ICG fluorescence imaging was useful for detecting superficially located small HCCs and confirming that these lesions had been removed with sufficient surgical margins [134].

Although liver cancer detection using ICG has already been proven to be feasible for clinical use, the true mechanism by which ICG accumulates in cancer-affected organs remains uncertain. In another study, Ishizawa et al. proved that uptake of ICG in differentiated HCC cells is mediated by Na+/taurocholate cotransporting polypeptide (NTCP) and organic aniontransporting polypeptide 8 (OATP8) in bile duct disorders, causing ICG to adhere to cancerous tissues. This enables highly sensitive detection of HCC via intraoperative ICG fluorescence imaging [135]. ICG-fluorescent imaging prior to resection recognized 21 out of 41 HCCs (51%), while all of the 16 metastases could be extensively characterized. This dye has further been successfully applied for the real-time differentiation of minute and grossly non-identifiable liver cancers [133].

**Clinical applications using tumor-specific agents**

ICG has FDA approval for many clinical applications. However, its inability to precisely and selectively target certain tumors and tissues limits its usefulness for many applications of intraoperative tumor detection. In 2011, European researchers reported intraoperative ovarian cancer detection by a folate receptor-$\alpha$ targeting fluorescent agent [7]. This method highlighted its potential applications in patients with ovarian cancer for improved intraoperative staging and more radical cytoreductive surgery. However, larger international multicenter studies using standardized, uniformly calibrated FMI systems are needed to further confirm the diagnostic (accuracy, sensitivity, and specificity) and therapeutic value of the reported approach in a larger series of patients. Results from studies of intraoperative surgical navigation system applications are shown in figure 2.

With regard to early stage tumor targeting agents, Sturm et al. presented a tumor-specific ASY*-FITC probe that was used in early stage esophageal adenocarcinoma detection during surgery. Although the detection was challenging because of the premalignant lesions’ flat appearance, 75% sensitivity and 97% specificity was achieved in this first in vivo human study. The results showed that this
targeted imaging agent was safe and could be useful for guiding tissue biopsy and for early detection of esophageal neoplasia and potentially other cancers of epithelial origin [136].

Precise and specific targeting of tumors by selective detection agents would also be of high clinical value. Report on urokinase plasminogen activator (urokinase plasminogen activator (uPA)) labeled with NIR dye supports the potential of tumor margin imaging and theranostic for image-guided surgery [137]. A few targeted antibodies for various tumors and tumor markers have been clinically approved, such as bevacizumab against VEGF, cetuximab against EGFR, and trastuzumab against HER2 [138]. A phase 1 clinical trial on breast cancer intraoperative visualization with bevacizumab conjugated to the IRDye 800CW has been approved and is undergoing trials [139]. In order to widely apply the intraoperative surgical navigation technology, clinical approved radionuclides using Cerenkov-induced fluorescence imaging can also be an effective method for intraoperative visualization of radiolabeled contrast agents [140]. Radiolabeled tumor-targeting peptides for molecular imaging, such as targeting integrin αvβ3, have been successfully translated into the clinical setting and showed potential for NIR fluorescence imaging [141]. Currently, these advanced systems and agents are available to research-oriented clinical trials; hence, surgeons are verifying this method and extending their application.

**Preclinical Studies with Clinical Translational Potential**

Recently, the non-targeted fluorescent dye ICG was adopted for clinical use after FDA approval. Current research focuses on increasing the availability of novel, fluorescently labeled agents to identify crucial landmarks, including: tumor margins, lymph nodes, and vital structures. Though many agents have proven their potential for clinical translation, profiles should first be considered before clinical use. Although many contrast agents have superior effectiveness in cancer detection, they may not be safe in patients and achieve FDA approval. Thus, satisfying safety profile requirements and the financial costs of clinical trials are challenges to the future approval of additional FMI agents.

Many promising imaging agents for different types of tumor targeting applications with clinical translational potential have been reported. Re-

![Figure 2. Clinical application results using surgical navigation systems. a) SPY imaging demonstrates perfusion to the thumb, index, and middle fingers via scanning with indocyanine green[142]. b) Instructions for the Artemis Handheld system, c) Intraoperative NIR fluorescent image of the Fluobeam camera system[143] d) fluorescent signal and/or blue color of lymph nodes detected by PDE[144], e) Single SLN identification by the FLARE system [10], f) In vivo fluorescence imaging of a lymph node detected by the Multispectral FMI system [17], g) ICG-guided intraoperative detection and resection of the SLN in humans by the surgical navigation system GXMI Navigator [60].](image)
searchers have developed “smart” agents, which can for instance target tumor cells, tumor angiogenesis, or the tumor microenvironment. These “smart” agents specifically target either through conjugation with tumor-specific antibodies [145-147], nanobodies [148], aptamers, or peptides with high affinity binding for proteins on the cell surface [149]; or through the precise localization and amplification of enzymes for fluorescence activation [41, 150-154].

In order to provide molecularly specific detection of cancer cells, NIR fluorescent agents are usually conjugated with a specific targeting ligand or monoclonal antibody targeting tumor cell receptors, such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER2), or vascular endothelial growth factor (VEGF) receptor [155-157]. Heath et al. conjugated the fluorescent dye (IRDye800CW) to a monoclonal antibody targeting EGFR to detect head and neck squamous cell carcinoma (HNSCC) in preclinical models. The specificity of tumor detection was confirmed by histology and immunohistochemistry (n = 25 of 25). The results demonstrate the feasibility of detection and dissection of HNSCC using this probe in clinical settings [158].

Sano et al. synthesized two probes, AlexaFluor680 conjugated panitumumab to target EGFR and ICG conjugated trastuzumab to target against HER2, to evaluate the feasibility of specifically detecting breast cancer cells in vitro and in vivo. The results showed specific expression in two breast tumor cell lines, MDA-MB-468 (EGFR+/HER2-) and 3T3/HER2 (EGFR-/HER2+), by the two activatable fluorescent probes. By using this method, intraoperative breast cancer molecular subtype classification can be realized [159]. For example, Scheltinga et al. labeled Zr-89 in the anti-VEGF antibody bevacizumab and anti-HER2 antibody trastuzumab with IRDye 800CW. Tumor uptake of the probes was determined in xenograft mice with radioactive counterparts for PET. Submillimeter level tumor lesions were also detected by real-time intraoperative FMI imaging method and confirmed by histology, immunohistochemistry, and fluorescence microscopy analyses [160]. The prospects of these cancer cell-specific imaging methods are encouraging for their future clinical translation to intraoperative image-guided surgery.

For the imaging of tumor angiogenesis, the molecule αvβ3 integrin is widely used [161], as it targets neovascularization at the tumor sites and can be visualized by conjugating it to an NIR fluorescent dye such as Cy 5.5 or IRdye800CW. Cyclic arginine-glycine-aspartate (RGD) is the commonly used ligand for targeting αvβ3. However, the existing drawback of RGD is its short blood circulation half-life, which greatly compromises its targeting efficacy. Chen et al. have solved this problem with a cyclic peptide. The c(RGDyK) and an organic dye (IRDye800 or Cy5.5) were covalently bound to human serum albumin (HSA), which improved the robustness of RGD targeting. Histology indicated that tumor vascular binding initially occurred where both tumor blood vessels and cell integrin were bound in vivo. This method may also be applied to other peptide-based probes that can be combined with HSA or other molecules for long-lasting tumor contrast and enhanced pharmacokinetics [162]. As further example, quantum dots (Qdots) possess excellent brightness, photostability, monodispersity, and fluorescent yield [163]. Li et al. synthesized cyclic RGD to the surface of NIR CdTe Qdots. Image-guided surgery was accomplished successfully with clear tumor margins visualization intraoperatively, demonstrating their potential for intraoperative tumor dissection [164].

The activatable strategy for the imaging tumor microenvironment takes advantage of the differences between tumor cells and normal cells, such as specific enzymes, pH value, temperature and other stimulations [165]. In vitro, these activatable probes are either not fluorescent or have little fluorescence due to the quenching effects of these conditions; however, after specific activation at the target site in vivo, they achieve strong fluorescence through the effect of dequenching. When the probes are activated, they exhibit high SBR compared to a constitutively fluorescent agent [19, 20, 166-173].

In order to improve SBR effectiveness, Elamprakash et al. designed a ratiometric activatable cell-penetrating peptide (RACPP) and coupled it with the fluorescence resonance energy transfer (FRET) technique using Cy5 and Cy7 dyes. Such ratiometric imaging increased SBR 40-fold and provided an accelerated and quantifiable metric to identify primary tumors and metastases in liver and lymph nodes with increased sensitivity and specificity [20].

For the purpose of real-time imaging, Zhu et al. discovered a method, which in a small animal model is able to boost fluorescent signals upon protease cleavage of the target molecule approximately half an hour post-injection. A strong fluorescent signal is then sustained for up to 24 hours. This particular method can identify any target protease with a specific peptide substrate and can adapt to a variety of real-time imaging applications, such as in vivo drug screening, drug efficacy trials, disease onset monitoring, and animal model development [166].

In order to improve sensitivity, Myochin et al. derived a new strategy to obtain a NIR fluorescence probe that can be rapidly activated by extracellular MMP. This design can be applied to develop a range
of more sensitive and rapidly responsive NIR fluorescent probes not only for MMP activity, but also for other proteases [168]. However, the major limitation for the clinical application of new NIR fluorescent agents is that each fluorophore-ligand conjugate must receive regulatory approval separately, which is costly and time consuming [174].

Radionuclide probes are FDA approved and widely used clinically. These agents can be used to generate Cerenkov luminescence (CL), which is light generated when charged particles exceed the speed of light in a dielectric medium. Recently, the feasibility of CL imaging in patients undergoing diagnostic $^{18}$F-FDG scans to detect nodal disease has been validated in clinical experiments [140]. This means that optical methods for the detection of numerous radionuclide probes can be applied in clinical practice. In addition to enabling Cerenkov optical imaging for intraoperative clinical use, tumor-specific clinical applied radiotracers have synergistic advantages for PET-based diagnostics and therapeutics. Thorek et al. reported that disease markers were detected using nanoparticles to produce secondary Cerenkov-induced fluorescence and could be applied to monitor other markers, representing a shift toward activatable nuclear medical agents [175]. New approaches using clinical PET tracers to produce secondary Cerenkov-induced fluorescence provide us with the opportunity to adopt their use for surgical applications [175]. These FDA approved radioactive agents expand the range of applications available during surgery [176, 177].

Conclusions and Perspectives

Precise medical diagnosis and treatment will approved the ability of surgeons to treat cancer. Intraoperative image-guided cancer surgery using FMI technology may provide the most valuable goal for addressing diseased and abnormal tissues in surgical practice. White-light reflectance supplies insufficient visual information between the tumor and normal tissue, whereas fluorescence can provide additional information to potentially prevent cancer persistence or recurrence, and unacceptable morbidity. Although FMI technology has been of substantial benefit to patient outcomes, much more work is necessary for clinical translation of the rapidly expanding number of targeted agents and imaging systems currently in the research pipeline.

Intraoperative FMI has good performance in clinical applications, and many patients benefit from this method. As penetration depth is a challenge in optical imaging, intraoperative multimodal data fusion (such as adding rescanned CT and/or ultrasonography) provides possible solutions [45, 178, 179]. These techniques will augment the tools for accurate preoperative diagnosis, such as radiography, CT, MRI, and PET, which will provide important aid in advancing intraoperative FMI detection.

Some preclinical results using the NIR imaging technique combined with other imaging methods such as ultrasonography [45], MRI [180] and X-ray CT [178] compensate for the depth issues and have already demonstrated the possibility of using the FMI guided multi-modality method to precisely excise tumors. Zhang et al. declared that tumor metastatic lymph nodes and reactive lymph nodes located in deep-seated area were distinguished with diffusion-weighted and super-paramagnetic iron oxide enhanced MR imaging [181]. Visualization of tumor draining SLNs at distant depths using NIR, MR and PET triple-modal imaging methods was done in a 4T1 tumor metastasis model and provided helpful guidance for SLN mapping and tumor metastasis diagnosis thereby revealing its potential clinical utility [182]. With breakthroughs in computer-aided treatment technology, multi-modality image registration with optical imaging methods has achieved clinical use in intraoperative applications. Through the development of intraoperative image-guided agents and imaging systems in cancer surgery, FMI technology will extend other modality imaging methods into the clinic empowering surgeons to improve patient outcomes.

Finally, it is important for surgeons to incorporate intraoperative surgical navigation technology into their practice. Along with innovation in fluorescent agent development, there is a parallel path of innovation in the field of instrumentation that will extend the traditional view of surgery. Although these advances in FMI navigation have the potential to improve the surgical paradigm, there is the remaining challenge of defining clinical end points that will most benefit surgeons and patients utilizing these molecular navigation techniques. Minimally invasive surgical techniques have altered surgical practices in a way that coincides with and complements these promising technological advances. Minimally invasive surgical modalities have limited the handling of tissues, forcing surgeons to rely more heavily on tissue visualization. For open surgeries, real time improvement in visual differentiation between dissimilar tissue types during surgery will be particularly advantageous. With the development of imaging techniques and contrasts, FMI method can provide powerful assistance to theranostics for patients. Significant advances in intraoperative imaging-guided cancer surgery are expected in the next few years.

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Competing Interests

The authors have declared that no competing interest exists.

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