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Molecular Imaging for Cancer Diagnosis and Surgery

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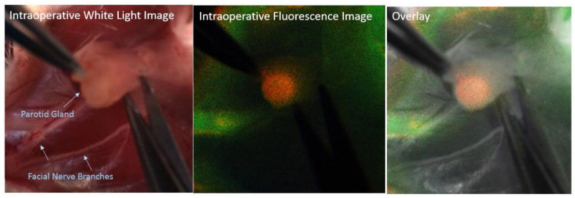
Abstract

Novel molecular imaging techniques have the potential to significantly enhance the diagnostic and therapeutic approaches for cancer treatment. For solid tumors in particular, novel molecular enhancers for imaging modalities such as US, CT, MRI and PET may facilitate earlier and more accurate diagnosis and staging which are prerequisites for successful surgical therapy. Enzymatically activatable “smart” molecular MRI probes seem particularly promising because of their potential to image tumors before and after surgical removal without re-administration of the probe to evaluate completeness of surgical resection. Furthermore, the use of “smart” MR probes as part of screening programs may enable detection of small tumors throughout the body in at-risk patient populations. Dual labeling of molecular MR probes with fluorescent dyes can add real time intraoperative guidance facilitating complete tumor resection and preservation of important structures. A truly theranostic approach with the further addition of therapeutic agents to the molecular probe for adjuvant therapy is conceivable for the future.

Graphical Abstract



Keywords

Molecular Imaging; Cancer diagnosis; Fluorescence guided surgery

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1. Introduction

In public discussion, improved drug treatment is often perceived as the main driver in the fight against cancer. However, in the case of solid tumors, early detection has to be considered equally, if not more important for successful treatment because it enables a surgical, curative approach. Surgery is usually limited to tumors detected at an early stage and outcomes decrease significantly once primary surgery is not a treatment option any more. For example, according to 2010 National Cancer Database (NCDB) data, 60% of stage I non-small cell lung cancer (NSCLC) patients had cancer removal surgery as their primary treatment, compared to just 6% diagnosed at stage III [1]. The 5-year survival rate for NSCLC patients whose cancer was surgically resected is 60 - 80% for stage I and 40 -50% for stage II [2]. Concurrently, non-resectable stage III NSCLC treated with chemotherapy is associated with 2-year survival rates of less than 20% [3], emphasizing the importance of early detection and subsequent surgical removal.

Over the past decades, substantial efforts have been made to detect malignancies at an earlier state. Much of the progress made in cancer diagnostics and staging can be attributed to technical advances in ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) which are essential for providing anatomic details for solid cancers [4]. Molecular imaging techniques may very well have the potential to improve every aspect of cancer care by opening up entirely new possibilities for the early detection and the effective treatment of cancer, both of which are essential to successfully fight the disease. Commonly and somewhat unspectacularly, molecular imaging is defined as non-invasive imaging of cellular and sub-cellular events [5]. More specifically, oncologic molecular imaging is based on highlighting distinctive molecular characteristics of malignant cells. Over the past years the genetically determined production of biomolecules by cancer cells has been extensively studied and characterized and individual expression profiles have been defined for certain types of cancer [6, 7]. Molecular imaging probes target and highlight these specific characteristics which can be exhibited either directly in, or on, individual malignant cells or in the surrounding extracellular matrix and cells in the vicinity, such as T cells, macrophages, dendritic cells, fibroblasts or endothelial cells [4].

Currently molecular imaging strategies for all major whole body imaging modalities for cancer diagnosis and staging as well as molecular imaging probes for optical imaging in cancer surgery are being developed [8, 9]. These strategies give US, CT and MR an entirely new dimension by expanding morphological imaging to a cellular, functional level. Selective depiction of cellular properties and their microenvironments characteristic for the malignant state will enable earlier detection, assessment of aggressiveness and lead to a more personalized treatment approach. Today, many clinicians still primarily associate molecular imaging with positron emission tomography (PET). Indeed, PET imaging with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) depicts the metabolic discrepancies between malignant and healthy cells, making PET the first “true” and most widespread molecular imaging modality. However, its high cost, use of ionizing radiation, and relatively low spatial resolution somewhat restrict its potential. Therefore, molecular imaging with higher resolution modalities, especially MR, is gaining increasing attention. Aside from the whole body imaging applications, molecular imaging probes have also been adapted for optical

imaging and can provide intraoperative guidance for cancer surgery. Ideally, molecular imaging probes will allow for earlier diagnostic imaging of solid cancers as well as facilitating better surgical treatment in the future, leading to an overall improved outcome. This review aims to outline relevant molecular imaging applications currently available or in development for the diagnosis, staging (CT, PET, US and MRI) and surgical treatment of cancers.

2. Molecular Imaging Applications in Cancer Diagnosis and Staging

2.1. New Levels of Cancer Diagnosis and Staging

Based on their inherent characteristic of making functional attributes of malignant cells visible, molecular imaging techniques have the potential to enhance cancer diagnosis and staging on multiple levels, most notably tumor detection and characterization. While US, CT and MR imaging technology is continuously advancing, tumor detection today is still largely performed based on anatomical characteristics. Molecular imaging applications can make properties of carcinogenesis visible at much earlier time points because alterations on the cellular level are targeted and can potentially be detected as soon as they occur. For example, abnormalities in malignant cells' glucose metabolism occur at very early time points in carcinogenesis [9, 10]. PET imaging with ^{18}F-FDG allows clinicians to detect those changes, although specificity and resolution for this imaging modality are limited. To truly exploit the potential of molecular imaging, e.g. performing regular whole body screening scans for at-risk patients to detect smallest malignant lesions, will depend on the development of more specific molecular imaging probes that target pathologic characteristics, ideally for imaging modalities without radiation exposure for the patient.

Tumor characterization without the need for invasive procedures such as biopsies or even surgery is considered another key feature molecular imaging adds to cancer diagnosis. While differentiation between benign and malignant tumors on conventional CT and MR scans based on morphologic characteristics can be difficult, molecular imaging allows for a much better assessment of aggressiveness because functional properties of malignant cells are visualized [11]. It has also been shown that a PET imaged decline in ^{18}F-FDG uptake after treatment initiation correlates with patient outcome for certain cancers [12, 13], allowing for more accurate staging and re-staging, as well as drug response monitoring of patients with molecular imaging technology. Eventually, molecular imaging may also be able to determine the ideal treatment for individual patients. Highly specific visualization of the expression profiles of certain molecular markers, a "molecular phenotype" associated with patient outcomes at the time of cancer diagnosis may provide guidance to a truly personalized treatment approach [9, 14, 15].

2.2. Molecular Targeting Approaches

Molecular imaging probes for cancer diagnosis usually comprise a signaling component which is detectable by the respective imaging modality and a targeting element. The latter can be highly specific to detect a certain type of malignancy or be aimed at more general features of malignant physiology.

Imaging probe targets which are not specific for a certain type of malignancy are aimed at functional or phenotypic characteristics exhibited by many cancer variants. The aforementioned PET imaging with 18F-fluorodeoxyglucose (^{18}F-FDG) depicts the metabolic discrepancies between malignant and healthy cells by indicating increased glucose uptake by cancer cells. An example for a more cancer specific approach which however is also not limited to a certain type of malignancy is designing probes which attach to certain integrins which are highly expressed on tumor vascular endothelial cells, while being almost undetectable on normal blood vessels, making them a potential target for imaging during early cancer diagnosis [16-18]. Similarly, probes developed in our collaborative laboratories are targeted at extracellular matrix enzymes, specifically matrix metalloproteinases (MMPs) 2 and 9 which are expressed by a range of tumor cell lines [19-24].

Enzymatically activated molecular imaging probes have additional value because they possibly allow for a more in-depth imaging approach. The amount of contrast enhancement created correlates with tumor cells' development, growth and productivity. Most enzyme targeted probes are engineered to be cleaved by the molecule they target for activation, some others are catalyzed to undergo bond formation which shifts them into a contrast generating state [25].

Activatable molecular imaging probes are also referred to as "smart" probes because they only exhibit a contrast enhancing signal under certain conditions. Besides enzymes, triggers can also be environmental variances, such as certain pH levels. Using pH differences for the detection of malignancies has been pursued for a long time since pH is somewhat lower in tumor cells compared to healthy tissue because of increased glycolytic activity. Several approaches to visualize intracellular pH differences have been reported over the past three decades, including fluorescent pH indicators [26] and MR spectroscopy [27]. Others utilized green fluorescent protein (GFP) mutants' pH-dependent absorbance and fluorescence properties to detect pH changes in cells [28]. This particular technique is useful in animal models but has limited clinical translatability due to the necessity for gene therapy to introduce GFP into human cancers. More recent studies have also investigated gadolinium based pH-sensitive contrast agents for MR imaging [29, 30].

Specific molecular imaging probes are constructed to interact with biomolecules characteristic for one specific type or class of malignancy, such as prostate specific antigen (PSA) expressed on prostate cancer cells [31], or carcinoembryonic antigen (CEA) expression on pancreatic cancer cells [32, 33]. As antigen-antibody interactions typically have very high specificity [34], they have attracted significant attention for molecular targeting. However, their one-to-one binding relationship limit probe accumulation to the cancer and are usually not sufficient for whole body imaging approaches. Furthermore, intact antibodies raise some concerns given their relatively large size and potential immunogenicity [8] although these may be mitigated by engineered antibody particles [35].

Most molecular imaging probes are engineered for systemic administration. Compared to other application routes, such as oral or direct intra-tumoral application, systemic administration allows for in-depth tumor penetration via the vasculature as well as sufficient

washout time to eliminate nonspecific fluorescence from non-targeted tissues [36]. Systemic administration, in contrast to local delivery such as topical application or intratumoral injection, also allows detection of previously unrecognized tumor foci or metastases.

2.3. Molecular Imaging Applications for CT, PET and US

CT generates three dimensional reconstructions of patient anatomy based on differences in X-ray attenuation [37]. Due to its speed, high spatial resolution and relative cost-effectiveness, CT is the most commonly used imaging technique in cancer diagnosis today to detect morphological abnormalities. However, current CT technology has some limitations which make it a less than ideal application for molecular probes for the detection of solid cancers. Most importantly, soft-tissue contrast is limited without contrast agents. Currently used contrast agents are mostly based on iodinated molecules which effectively absorb X-rays. However, to achieve sufficient sensitivity, quantities in the range of several millimolar contrast agent concentrations are required. This is several orders of magnitude higher than gadolinium chelates for MR contrast which require micromolar concentrations due to superparamagnetic particles influencing water protons in a space around them of up to 50 times their own diameter [38]. CT contrast agents do not have this amplification ability, therefore a large amount of heavy molecules is required to achieve satisfactory sensitivity, raising toxicity concerns and so far limiting the development of iodine based CT molecular imaging probes for cancer.

More recently, the emergence of nanomaterials has revealed new possibilities for imaging cancer with targeted molecular imaging probes for CT. For example, bismuth sulfide nanoparticles provide more enhanced sensitivity than iodine based probes because of their higher atomic number while at the same time overcoming some additional limitations of iodine based contrast agents, such as their rapid excretion [39]. For certain clinical applications, these nanoparticle-based molecular imaging probes for CT have been shown to be promising, such as the detection of macrophages in atherosclerotic plaques, as well as imaging of macrophage-rich organs like the liver and the spleen [39, 40]. The development of future applications for cancer diagnosis will depend primarily on whether the required intrinsic concentrations of heavy elements can be further reduced.

Currently, CT imaging plays an important role in molecular imaging by providing additional anatomical resolution for PET imaging. A PET scanner specifically detects photons which are generated by positron/electron encounter which occurs directly after positron emission by radionuclides [41]. The most relevant biomarker for PET is the radionuclide ^{18}F-FDG, which is incorporated into malignant cells by glucose transporters which are overexpressed by cancer cells [42, 43]. ^{18}F-FDG is phosphorylated by hexokinase, providing an indication of elevated glucose consumption or metabolism [11, 44, 45]. Uptake is increased in several types of cancer, including those of the lung, the GI-tract, the head and neck, as well as cervical, ovarian and breast cancers [46]. For cancers which do not exhibit increased ^{18}F-FDG uptake, multiple new tracers for PET imaging are being developed. Among them are tracers with the potential to highlight neuroendocrine tumors as well as bone metastases in prostate cancer patients [47]. While PET, PET/CT and increasingly also PET/MRI are well established in molecular imaging for cancer diagnosis, they do have some limitations. Aside

from the radioactivity the patient is exposed to, specificity is somewhat limited by the characteristics of the molecular tracers used for PET imaging which are not specifically targeted at cancer cells [48]. Other areas in the body with an increased glucose uptake, such as inflammation, or hyperplastic bone marrow also show an increased ^{18}F-FDG uptake which can lead to false positive results. Nevertheless, PET imaging is the only true molecular imaging modality for the diagnosis and staging of cancer that already plays an important role in today's clinical routine [11].

Ultrasound (US) imaging technology is based on the detection of reflected sound pulses. The pulses are generated by a transducer and propagate into tissue where they are reflected in patterns depending on the tissues' density and compressibility [49]. US has many advantageous qualities including high spatial and temporal resolution, real time imaging, lack of radiation, portability and low cost [50], so there has been significant effort to develop molecularly targeted probes for this modality. In general, there are two main types of US imaging probes, namely microbubbles and non microbubble agents [51]. Microbubbles are gas liquid emulsions with a gas core of about 1 – 4 μm in size. Their core causes a high echogenic response resulting in a high contrast to tissue background ratio on the ultrasonic image [50]. Depending on the desired target of the probe, the bubbles' shell composition varies. Microbubbles do not leave the vasculature because of their size. Therefore, as molecular imaging probes for cancer diagnosis their main indication is to image angiogenic processes by targeting specific markers on tumor vascular endothelial cells [8, 50]. Studies in mice and rats have shown promising results for different types of cancer. For prostate cancer, microbubbles targeting vascular endothelial growth factor receptor 2 (VEGFR2) have been successfully tested [52, 53]. Contrast may be further enhanced by using dual-targeted microbubbles, which have a higher binding efficiency, as shown by Willmann et al. who targeted $\alpha v\beta 3$ integrin as well as VEGFR2 [54]. Since many newer cancer drugs target angiogenic activity, US imaging with molecular microbubble based probes may be of interest not just for cancer diagnosis but could also have a valuable role in monitoring the effect of anti-cancer therapy [55].

Non microbubble molecular probes are in the 10 to 10,000 nanometer range and consist of liquid or solid colloids [51]. They may have the potential to further expand the possibilities of using US as a modality for cancer diagnosis because their size allows them to leave the vasculature. The aforementioned augmented permeability of tumor vasculature compared to healthy tissue, known as the Enhanced Permeability and Retention (EPR) effect [56, 57], opens up the perspective of creating US imaging agents which are small enough to cross the leaky, defective vasculature of the tumor while not leaving the vasculature in healthy tissue. The potential of this approach is not limited to molecularly targeted US tumor imaging applications for cancer diagnosis but could play an additional role in the treatment of solid tumors, as well. These probes could deliver highly toxic anti-cancer drugs to their intended destination utilizing the EPR effect while sparing healthy tissue from adverse drug effects [50]. Ideally, the successful accumulation of drug loaded nanoparticles is then visualized via US. Recently developed multifunctional drug carrying nanoparticles do not only deliver drugs to the intended destination but are constructed to release the active anti-cancer agent when tumor directed ultrasound is applied [58, 59]. However, despite promising results for such molecular probes capitalizing on the EPR effect in fast growing implanted tumors in

rodent models, limitations for the applications in human clinical tumors remain. Here, there is a great heterogeneity among different tumors and not all malignant tissue consistently exhibits the EPR effect, particularly necrotic or other hypovascular areas of larger tumors or metastases [60]. Furthermore, for early cancer diagnosis, their small size is also non microbubble molecular probes' principal limitation because their acoustic reflectivity in certain tissues is only minimal and makes them difficult to visualize at low concentrations [50]. Nevertheless, the rapid developments being made in US technology as well as the aforementioned inherent advantages of US imaging make it likely that molecular imaging applications for US will become a clinical reality. It's availability and low cost make molecular US make especially intriguing for repeated visualization of malignancies, for example to monitor the effects of anti-cancer therapies [61].

2.4. Molecular Imaging Applications for MRI

MRI technology is based in principle on creating a magnetic field surrounding the patient which aligns magnetic dipoles such as hydrogen atoms in water. After a temporary radiofrequency pulse changes the alignment of the dipoles they return to their baseline orientation at a rate which is determined by their physiochemical environment and which is detected and translated into a MR signal [62, 63]. MRI offers high spatial resolution and excellent anatomical detail without any exposure to radiation for the patient making it an essential modality for the detection of solid cancers. More recent technical advances like the introduction of stronger magnets and parallel imaging already allow for a certain degree of functional MR imaging in cancer diagnostics even without the application of molecular probes. For example, diffusion-weighted (DW) MR imaging exploits the fact that in most cancer types, the diffusion of water molecules is slower than in healthy tissue to generate contrast enhancement [9]. Studies evaluating the use of DW MRI compared to T2-weighted imaging alone for the detection of prostate cancer have shown some promise [64-66] and whole-body DW MRI has been shown to be as accurate as PET/CT for the detection of lung cancer metastases [67]. It is still somewhat unclear, though, whether there is an immediate clinical benefit for the earlier detection of cancer compared to T2-weighted imaging at this technical stage. Studies do show that DW MRI can already be used reliably to determine the aggressiveness of certain malignancies [68].

In light of the inherent benefits of MR imaging, the development of MR molecular imaging techniques for the detection of solid cancers has recently quickly progressed. One difficulty which has to be taken into consideration when developing molecular probes for MRI is the relatively low sensitivity in depicting most contrast agents. MRI is several orders of magnitude less sensitive than bioluminescent optical or radionuclide imaging techniques which require reporter probe concentrations as low 10^{-12} M. For MRI imaging, larger amounts of the contrast agent have to be retained at the target site [62] and amplification strategies are particularly crucial to detect molecular probes aimed at sparsely expressed biomolecules.

Standard contrast agents are engineered to enhance MRI signal either in T1 or T2 weighted sequences. Contrast agents used in T2-weighted images are usually based on various forms of iron oxide nanoparticles, which change the relaxation rate of water protons detected by

MRI. Superparamagnetic iron oxide nanoparticles (SPIO) used today can be conjugated with specific targeting ligands and serve as molecular imaging probes [69]. SPIOs have a polymer coating which may be modified to specifically target receptor molecules or proteins [70]. They have been successfully used to detect lymph node metastases in prostate cancer [71, 72], as well as angiogenic activity in melanoma bearing mice [73]. Imaging tumor cells with SPIOs faces some challenges because the nanoparticles tend to remain in the vasculature and are metabolized by the reticuloendothelial system. Nevertheless, studies with SPIOs have shown success targeting malignant cells expressing elevated levels of transferrin receptors [74] and especially folate receptors [75, 76], which are overexpressed on certain types of cancer, such as ovarian and breast cancers [77, 78]. While recent technical advances such as the creation of smaller nanoparticles have opened up interesting perspectives for future applications, the use of T2-weighted molecular imaging probes has the inherent disadvantage of providing negative contrast enhancement (signal is reduced in T2 weighted images and the target structure is darker). This generally limits their use in low signal regions of the body because negative contrast can oftentimes not be distinguished from a void in the image [79]. Cancer diagnosis and staging in particular may also be hampered in areas of the body where magnetic susceptibility artifacts can lead to hypo-intensities. Potential causes include the presence of air, such as in the lungs, flow-related signal losses or calcification [80].

To enhance contrast in T1 weighted MRI sequences, by far the most commonly used contrast agents are paramagnetic gadolinium (Gd) chelates. For the development of targeted and especially activatable, “smart” MRI probes, paramagnetic chelates are better suited than nanoparticle-based probes because of their versatility in design and controllability allowing them to undergo various intramolecular transitions and intermolecular interactions [25]. Their detection threshold in the micromolar range [81] requires some adjustments for their use in molecular imaging applications and larger Gd payloads have been successfully added to the particles used as molecular imaging probes [82].

In recent years, several targeted Gd-based molecular MRI probes have been reported which are activatable by enzymatic cleavage. The specific targeted enzymes serve as a markers for certain diseases or conditions, for example esterases in macrophages which are elevated at sites of inflammation [83] or myeloperoxidase which has been linked to cardiovascular and neurological diseases [84]. To enhance the visualization of malignant processes, recent studies have focused on MMPs as enzymatic targets for activatable molecular probes. Elevated levels of MMPs are not only expressed by multiple tumor cell lines [19-24], but have also been shown correlate with the invasiveness of cancer cells, particularly MMP 2 and 9 [85]. Several approaches to ensure sufficient accumulation of the enzymatically cleaved molecular imaging probe have been reported. Lepage et al. designed two different MRI contrast agents which upon cleavage by MMP 2 and 7 undergo a solubility switch leading an accumulation of the imaging probe in areas of high MMP concentration [86, 87]. Other studies have implemented the opposite principle, where cleavage of the Gd-based MR imaging probe by MMPs leads to an increased solubility and washout of the probe [88]. In yet another approach, a molecular imaging probe was created to switch from a stable low-

relaxivity state to a highly surface charged, aggregative state upon cleavage by MMP 9 *in vitro* [89].

While the above named probes used protease cleavage to produce contrast in the vicinity of malignant cells, we have adopted a strategy in which MMP–cleavage produces adhesion and uptake into the tumor by attaching Gd payloads to activatable cell penetrating peptides (ACPPs). Cell penetrating peptides (CPPs) are able to deliver cargoes significantly greater than their own weight into cells without requiring specific receptors [90]. Several mechanisms have been identified which deliver the peptides into the cells but are still poorly understood, however most seem to require contact between the CPPs and negative residues on the cell surface [91]. ACPPs are modified from CPPs by attaching them to neutralizing polyanions with protease-cleavable linkers. Upon cleavage, in this case by MMP 2 and 9, the polycation is released and is able to adhere to and penetrate cells in the immediate vicinity of the enzyme. Previous studies conducted in our laboratory showed that ACPPs can penetrate deep into tumor nodules [92-95]. To increase circulation time of ACPPs and hereby improve tumor to background contrast, we added a large molecular weight carrier to ACPPs [94]. The polyamidoamine (PAMAM) dendrimers chosen as the macromolecules allow for the attachment of multiple Gd payloads per ACPP (ACPP dendrimers – ACPPD) and even dual labeling with Gd and fluorescence for both MR and optical intraoperative imaging (see next section) [96].

MRI results in mice showed that uptake in tumors was up to 50μM Gd and T1 contrast in sub-centimeter foci of cancer was detectable for several days [95]. This suggests that using MMP-cleavable ACPPDs as molecular imaging probes for MRI may be beneficial to image tumors and metastases as well as positive lymph nodes which may otherwise not be detected by MRI. Especially for small invasive malignancies Gd loaded ACPPs may add value to the diagnostic and staging process. The long retention time in cancer cells is especially advantageous for the postoperative evaluation of patients with solid cancers. Clinical evaluation of surgical margins using MRI with standard Gd contrast agent administered after surgery is complicated by increased Gd uptake due to postoperative changes. Gd loaded ACPPDs can be injected well before the surgery, which enables the imaging probe to clear from circulation before the procedure. Signal detected several hours or even days after the procedure can most likely be attributed to preoperative uptake into cancer cells and not intra- or postoperative tissue accumulation and gives physicians the opportunity to assess postoperative surgical margins. Furthermore, thanks to the sensitivity of Gd loaded ACPPDs enabling the detection of small tumors, patients who are at a high risk of getting cancer for genetic or other reasons could receive whole body MRI scans using this molecular imaging probe at regular intervals to detect newly developing tumors as early as possible.

3. Molecular Imaging in Cancer Surgery

Advances in early diagnosis will allow more patients to pursue a surgical cure for cancer treatment. The main goal of cancer surgery is to identify and remove as much diseased tissue as possible while limiting damage to healthy tissue and structures. The standard of care in oncologic surgery currently relies on white light reflectance which limits the differentiation between normal and diseased tissue to a narrow palette between tissue colors

and texture. Molecular imaging not only has the potential to improve early detection and staging of malignancies as described above but can also provide valuable intraoperative guidance to the operative surgeon to improve outcome [96-98].

3.1. Visualization of Cancerous Tissue

In cancer surgery it is of utmost importance to exactly identify the extent of the malignancy because the presence or absence of tumor cells at the cancer site after surgical removal (i.e. “surgical margin”) is a decisive factor in determining the success or failure of the curative approach and is often used to determine necessity for adjuvant therapy. Positive margins (the presence of residual cancer cells at the surgical margin) are a negative prognostic indicator for many solid cancers, including those of the head and neck, lung, breast, colon and urogenital tract [99-105]. Currently, the surgical margins are often evaluated by immediate intraoperative analysis of samples [100], which not only extends the time of operation but may also give incomplete results depending on the quality of the samples and the limited sampling of the tissue, resulting in positive margins. Thus, endogenous and extrinsic approaches have been developed over the past decades to facilitate the identification of diseased versus healthy tissues intraoperatively, almost all of which are applied to optical imaging techniques.

Microscopic imaging systems have been developed which enable the differentiation between healthy and diseased tissue based on endogenous contrast or autofluorescence of certain malignancies in vivo to identify critical surgical margins [106-110]. While the general approach of eliminating extrinsic contrast agents entirely is enticing, high resolution of the diseased tissue using endogenous contrast is limited to a very small field of view which makes surveying the entire excision margin intraoperatively unrealistic.

Extrinsic approaches for optical imaging are a more promising alternative. They are primarily based on fluorescent dyes which can be detected at relatively low probe concentrations; in addition, the fluorescent signal can be integrated into the white light image, enabling real-time intraoperative visualization. Indocyanine green (ICG) was one of the first fluorescent dyes tested for intraoperative application in glioma surgery [111]. ICG is an untargeted dye which clears from the tumor at a different rate than from the surrounding tissue and can be visualized green under a specialized microscope [112]. ICG has since shown promise for intraoperative sentinel lymph node mapping [113] however its value for intraoperative detection of other solid tumor margins is limited [114].

To reliably visualize different types of cancers and enhance labeling specificity, several attempts have been made to create targeted probes for intraoperative imaging. So far, 5-aminolevulinic acid (5-ALA) is the only imaging probe which has been shown to improve tumor free survival after surgery [97]. 5-ALA is not fluorescent itself but elicits synthesis of fluorescent porphyrins in certain cancerous tissues [115]. It leads to accumulation of porphyrins in malignant gliomas and it has been shown that 5-ALA can lead to more complete resections of contrast enhancing tumors [97, 116]. However, some concerns remain regarding the varying levels of protoporphyrin production for different stages of the disease [117], which might lead to a certain degree of heterogeneity in 5-ALA fluorescence.

Thus, for optimized intraoperative visualization, fluorescent molecular imaging probes targeting cancer specific biomolecules are required. Some promising results have been reported for fluorescently labeled probes targeting carcinoembryonic antigen (CEA) in pancreatic cancer models [32, 33], and prostate specific antigen (PSA) in prostate cancer models, both in mice [118]. Notably, folate receptor targeted fluorescent imaging probes have recently been successfully applied for the intraoperative detection of ovarian cancer in humans [98], and effective attempts to further improve folate receptor targeted imaging probes have been reported [119]. However, these strategies have the inherent disadvantage that one targeted biomolecule can only bind to one probe, limiting the amount of fluorescent markers that can be accumulated at the site. In addition, CEA and PSA targeted probes are directed at unique surface markers which limits their application to one type of malignancy. The previously describes ACPPs developed in our laboratories are targeted at the strongly cancer associated enzymes MMP2 and MMP9. One active enzyme can cleave many substrate ACPPs, also their size also allows them to penetrate into tumor nodules, enabling strong fluorescent signal in tumors [92, 94]. ACPPs fluorescently labeled with Cy5 and conjugated to dendrimers (ACPPDs) used to guide the resection of breast and melanoma cancers in mice decreased the incidence of positive surgical margins and increased tumor-free survival [96]. The amenability of ACPPDs for dual labeling with fluorescent Cy5 as well as for MRI makes them unique because the application of a single probe allows for pre-operative staging, intraoperative surgical guidance as well as postoperative evaluation.

3.2. Identification of Important Structures

While in cancer surgery the main focus has to be the identification and removal of all diseased tissue to cure the patient from an otherwise potentially lethal disease, it is nevertheless essential to preserve as much healthy tissue as possible, especially important structures like nerves, blood vessels and ureters. In the context of this review article it should be briefly mentioned that molecular imaging applications have been developed which can help surgeons identify specific tissues intraoperatively. For blood vessels [120-122] and ureters [123, 124], non-targeted, non-specific fluorescent probes have shown some promise because they can be injected directly into the desired structures; however, for nerve identification, targeted, specific and therefore truly molecular probes have to be developed. This could be especially useful for surgeons attempting to remove a tumor growing close to delicate, important nerves. Injuries to the facial nerve during parotid gland cancer surgeries [125, 126], the neurovascular bundle around the prostate gland during radical prostatectomies [127] or the recurrent laryngeal nerve during thyroid surgeries [128, 129] are common and can oftentimes be attributed to poor visualization under white light. Current intraoperative nerve monitoring modalities such as electromyographic monitoring (EMG) [129, 130] do not provide visual guidance and fluorescent dyes for anterograde or retrograde tracing only label one nerve fiber tract at a time at a very slow pace [131, 132]. Fluorescent molecular imaging probes selectively targeting nerve tissue have been shown to label all nerves in the body within a few hours after injection and nerve branches as small as 50 μm could be visualized in a mouse model [133, 134]. Administering these probes before cancer surgery could greatly aid identification and help prevent accidental injury.

4. Technical and Logistical Considerations

There is little doubt that molecular imaging technology has the potential to add substantial value to the diagnosis and surgical treatment of cancer. This section will address some substantial technical and logistical aspects which need to be taken into consideration on the path to wide-spread clinical use of molecular imaging probes. Key issues include probe design and imaging technology determining sensitivity and specificity which have been broached throughout this article and are briefly summarized, as well as regulatory considerations for the introduction of new imaging probes and instrumentation, especially for optical imaging applications. The latter have been discussed in great detail elsewhere [135-138], but should be mentioned for reasons of completeness.

Further improving the sensitivity of molecular imaging modalities will be essential for cancer diagnosis and staging in order to ultimately be able to detect even the smallest malignant lesions. US imaging with microbubble agents has been reported to provide picogram sensitivity *in vitro* [139], however, for whole boy imaging, PET imaging with radionuclides is still the most sensitive modality today; it outmatches MR imaging with molecular contrast agents and is several orders of magnitude more sensitive than molecular CT imaging. Further advances in molecular imaging probe design, such as attaching a larger amount of contrast agent (e.g. Gd chelates for MRI) per probe particle [82], or improved X-ray absorption by CT agents, as well as increasing probe accumulation in the tumor to name just some, will be necessary to increase sensitivity in the future. For MRI in particular, technological advances such as the introduction of MR scanners with higher field strengths may allow for the introduction of new classes of more sensitive contrast agents for molecular imaging applications, for example those based on chemical exchange saturation transfer (CEST) [140].

Specificity of molecular imaging is largely dependent on molecular imaging probe design. As discussed above, probes with varying target sensitivity/specificity are currently in use or being investigated. Current approaches with high sensitivity such as increased cell glucose metabolism or MMP upregulation are not cancer specific and can also be detected at sites of inflammation. Some highly disease specific probes on the other hand are engineered to target molecules, such as antigens which are only expressed on one type of cancer. From a clinical perspective, probes which specifically target properties exhibited by all cancers have value, e.g. for diagnostic screening test, as do imaging probes which target specific features of a certain type of cancer. Those might for example provide very detailed information about a patient’s individual prognosis and the suitable therapeutic approach. One way to increase specificity for these probes and eliminate signal generation in non-cancerous tissues is to potentially combine imaging modalities such as PET/CT, PET/MR or MR/US into a multimodal approach targeting different characteristics of the pathology in question [8].

When thinking about the development of imaging probes targeting a specific cancer characteristic it is important to keep in mind that while such a diagnostic tool might be of tremendous value to the patient group affected by the particular form of cancer, such specificity also leads to a significantly reduced market expectation for the imaging probe [137]. Molecular imaging probes are required to undergo the same FDA approval process as

drugs and it was estimated that it takes about a decade and $ 150 million for a new imaging agent to arrive on the market. At the same time, imaging agents designed for single or limited use are much less lucrative than therapeutic agents designed for daily or frequently repeated use. Revenues even for the best-selling morphological X-ray and MRI contrast agents are only in the range of a small specialty drug [136]. Therefore, developing molecular imaging probes with a broader targeting design might reduce the financial risk for pharmaceutical companies. For the future development of highly specific molecular imaging probes, pursuing a “theranostic” approach early in the developmental process might be promising. Including the possibility of attaching chemotherapeutic cargoes to the imaging probes which can be delivered directly and exclusively to the targeted malignant cells could be a viable path to ensure sufficient reimbursement [141].

For the application of molecular imaging probes in cancer surgery, instrumentation is an additional technical and financial barrier that needs to be taken into consideration. While molecular imaging probes for cancer diagnosis and staging mostly rely on already existing imaging instrumentation (US, CT, MRI) for visualization, in surgery, new devices need to be developed, approved by the FDA and purchased by medical institutions. For surgical procedures during which patient tissues are routinely viewed through an interface or displayed on a screen and cameras are already in place, the imaging hardware can potentially be added to existing instrumentation [36], facilitating the development of molecular imaging probes which can be applied in microscopic, endoscopic, laparoscopic and robotic surgery.

5. Conclusion and Outlook

Early detection, accurate staging and complete surgical removal are crucial in order to successfully treat and potentially cure patients with solid cancers. Molecular imaging techniques have the potential to play an important role in improving cancer diagnosis and treatment by expanding existing whole body imaging modalities to a functional, cellular level as well as enhancing intraoperative visualization of diseased and healthy tissues for surgeons. PET imaging has already established itself as an indispensable and truly molecular imaging modality in today’s clinical routine. However, currently developed molecular imaging probes for US and especially MRI may soon allow for a more specific detection and accurate visual enhancement of cancer cells while at the same time providing information about their invasiveness and biomolecular production profiles.

Targeted multimodality probes such as ACPPs conjugated to dendrimers loaded with long lasting MRI contrast agent as well as fluorescent dyes for intraoperative guidance are especially intriguing probes for molecular imaging. A one-time administration can potentially facilitate diagnosis and staging, surgical planning, intraoperative guidance and post-operative evaluation of the surgical procedure, promising considerable synergistic benefits. One additional future advancement might be the addition of specific chemotherapeutic cargoes to the targeted probes. Ideally, anti-cancer drugs could hereby be deposited directly into the target cells, making the molecular probes truly “theranostic” drugs (combining therapeutic and diagnostic properties). Due to the Gd labeling, the efficiency of drug delivery could be directly monitored by MRI imaging. Also, the exposure

of healthy cells in the body to the toxic anti-cancer drugs would be limited since cleavage by cancer specific enzymes is a prerequisite for cellular uptake.

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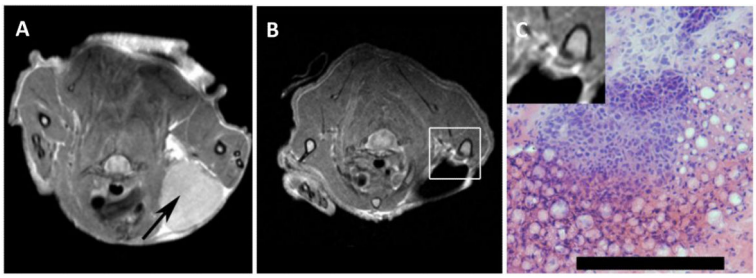

Figure 1.

Preoperative MRI image of a mouse injected with gadolinium and Cy5 dual-labeled ACPPD showing contrast uptake in tumor (A, black arrow pointing to the tumor). Repeat MRI (B) following tumor removal surgery showed a small area of tissue with increased gadolinium uptake (C inset, white arrowhead). Histological analysis confirmed the presence of cancer cells (C). Scale bar 100 μm. (Adapted and reprinted from reference 84.)

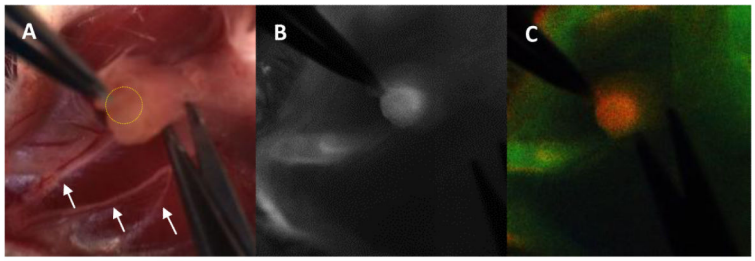

Figure 2. Fluorescent labeling facilitates intraoperative tumor identification

In a parotid gland cancer model of a mouse, no tumor is immediately apparent in the white light image (A, the yellow outline indicates the extent of the tumor, white arrows point to branches of the facial nerve). Under fluorescence guidance with a fluorescently labeled ACPP, the tumor and its extent are easily identifiable (B: fluorescent image, C: color overlay).

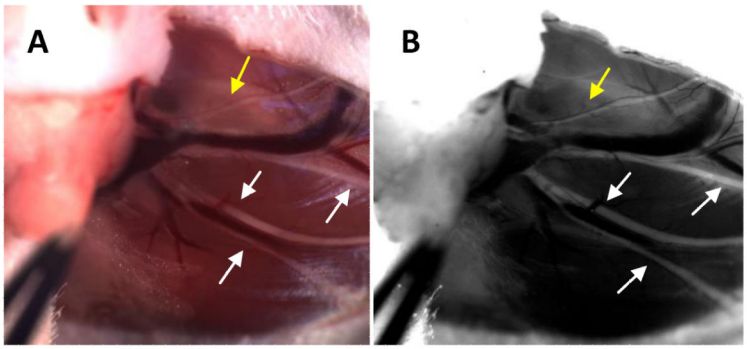

Figure 3. Enhanced nerve visualization

The identification of the branches of a right facial nerve of a mouse is enhanced by fluorescent molecular probe labeling (B) compared to the white light image (A). Large superficial branches (white arrows) can be easily identified even under white light alone, visualization of smaller branches is clearly improved by fluorescence imaging.

Table 1

Summary of the molecular imaging applications for cancer diagnosis and surgery discussed in this review article

The arrow in the left column indicates decreasing sensitivity. The list of signal generating agents and key targets reflects a selection based on the applications discussed and referenced in this review and is not exhaustive. Abbreviations: MI: Molecular Imaging; US: Ultrasonography; PET: Positron Emission Tomography; MRI: Magnetic Resonance Imaging; CT: Computed Tomography; CEST: Chemical Exchange Saturation Transfer CEA: Carcinoembryonic Antigen; PSA: Prostate-Specific Antigen; MMPs: Matrix Metalloproteinases; VEGFR: Vascular Endothelial Growth Factor Receptor; FDA: Food and Drug Administration; EMA: European Medicines Agency.

Sensitivity	Imaging Modality	Primary Signal Generating Agents	Key Targets	Status and Outlook for cancer diagnosis and therapy
	Optical	Fluorescent dyes Fluorescence inducing agents	Metabolic characteristics (e.g. porphyrine synthesis) Molecular markers (e.g. CEA, PSA, integrins) Enzymes (e.g. MMPs)	EMA approved, in phase III clinical trials for FDA approval: 5-aminolevulenic acid for intraoperative visualization of brain tumors Advantages: High sensitivity, can label anatomical structures (i.e. vessels, nerves, ureters) to differentiate from tumors Limitations: Limited depth penetration, additional Instrumentation required Outlook: Promising for cancer surgery and potential for photodynamic cleanup after surgery
	US	Microbubbles Nanoparticles	Molecular markers (e.g. integrins, VEGFR)	No FDA/EMA approved MI probes yet. Advantages: High sensitivity, lack of radiation Limitations: No whole body imaging Outlook: Promising for localized assessment of molecular target expression and therapy effectiveness
	PET	Radionuclides	Metabolic characteristics (e.g. glucose metabolism, phospholipid production) Molecular markers (e.g. androgen receptors)	FDA/EMA approved tracers: ^{18}F, ^{11}C, ^{68}Ga Advantages: Established in clinical practice, high sensitivity Limitations: Radiation exposure, low spatial resolution, limited specificity Outlook: PET/CT and PET/MR
	MRI	Gadolinium chelates (T1) Iron oxide nanoparticles (T2) CEST agents	Molecular markers (e.g. integrins, folate receptors, PSA) Enzymes (e.g. MMPs, myeloperoxidase)	No FDA/EMA approved MI probes yet. Advantages: High resolution, no radiation, functional imaging without imaging probes Limitations: Limited Sensitivity Outlook: Promising whole body imaging modality with improved imaging technology and probe design
	CT	Iodinated molecules X-ray absorbing nanoparticles	Molecular markers (e.g. integrins) Metabolic characteristics (e.g. glucose metabolism)	No FDA/EMA approved MI probes yet. Advantages: High resolution and speed Limitations: Low sensitivity, limited soft tissue contrast, radiation exposure Outlook: Dependent on development of new nanoparticle agents