

A Bright Future for Precision Medicine: Advances in Fluorescent Chemical Probe Design and Their Clinical Application

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The Precision Medicine Initiative aims to use advances in basic and clinical research to develop therapeutics that selectively target and kill cancer cells. Under the same doctrine of precision medicine, there is an equally important need to visualize these diseased cells to enable diagnosis, facilitate surgical resection, and monitor therapeutic response. Therefore, there is a great opportunity for chemists to develop chemically tractable probes that can image cancer in vivo. This review focuses on recent advances in the development of optical probes, as well as their current and future applications in the clinical management of cancer. The progress in probe development described here suggests that optical imaging is an important and rapidly developing field of study that encourages continued collaboration among chemists, biologists, and clinicians to further refine these tools for interventional surgical imaging, as well as for diagnostic and therapeutic applications.

Introduction

A growing body of basic and clinical research around precision medicine—harnessing biomedical tools to deliver tailored diagnostics and therapeutics for each patient—promises to revolutionize the way translational research and clinical medicine is conducted. In the field of oncology, advances in precision medicine will shift therapeutics away from untargeted chemotherapies that damage healthy cells in addition to cancerous cells, thereby causing serious side effects and poor quality of life. By exploiting specific mutations and pathways unique to cancer cells, therapies will instead target diseased cells while leaving healthy tissue unharmed. This will be a long-term battle, as researchers and clinicians work to discover markers and molecular signatures that subcategorize cancers into specific populations, enabling tailored treatments with improved response rates. In parallel with these therapeutic efforts, there is a significant need to develop tools that will allow necessary classifications of cancer subtypes to be made rapidly, accurately, and using minimally invasive methods. Furthermore, the development of agents that assess therapeutic response to guide treatment regimens is likely to alter patient outcomes in a significantly positive way.

With the launch of the Precision Medicine Initiative (PMI) by President Barack Obama in January 2015, the concept of personalized medicine evolved to encompass a wide range of determinants that impact health (Collins and Varmus, 2015; Obama, 2015). Genetic information remains an important component of decision making for the treatment of cancer, but the tenets of precision medicine are expanded to include a wider range of cellular and molecular analyses, environmental and lifestyle choices, and an emphasis on disease prevention. The concept of “precision” has particular resonance in the field of

surgical oncology, where the gold standard and first step in many patients’ treatment plans is surgery (Centers for Disease Control and Prevention, 2012; Howlader et al., 2014; Siegel et al., 2012). Precision at the interventional stage is increased by innovations in techniques and methodologies that help doctors and surgeons to better visualize each patient’s disease. One such breakthrough that is likely to have a broad impact on cancer management is the application of imaging tools that enable optical surgical guidance with molecular precision.

The primary challenge for surgical intervention in cancer treatment is finding effective ways to define the boundaries between the tumor and healthy surrounding tissue at the cellular level. Technologies to define the molecular edge between cancer and healthy tissue are designed to exploit characteristics of all cancers, or types of cancers, to precisely identify diseased tissues in the majority of patients. Furthermore, these technologies can light up critical structures to preserve, such as ureters and nerves (Park et al., 2014; Verbeek et al., 2014b; also reviewed in Orosco et al., 2013), that cause significant morbidity when accidentally damaged in surgery. Many of these technologies are already reaching clinical trials, and therefore have the potential to positively benefit patients in a shorter time frame than many of the precision therapeutics. In this way, imaging with optical guidance during surgery represents a technology complementary to the aims of precision medicine in diagnostics and therapeutics, and these technologies should be encompassed in our goals to improve our understanding of the prevention and treatment of human disease. Precision imaging holds additional promise, as many of the tools developed and optimized to image disease can be further harnessed to specifically deliver therapeutics to the target tissue of interest. Together, precision imaging in conjunction with other research aims under the

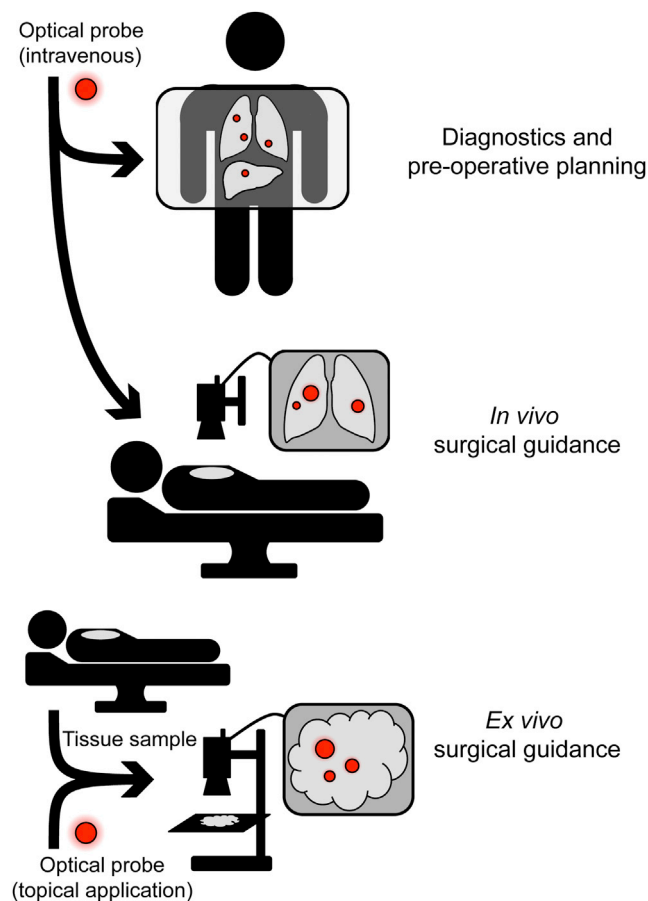


Figure 1. Current and Future Clinical Applications for Optical Chemical Probes

Top: future application of optical contrast agents includes intravenous administration for pre-operative diagnostics and planning. Middle: current use includes of optical contrast agents for in vivo surgical guidance. Bottom: topical probe labeling for ex vivo surgical guidance. Red circles denote optical probe accumulation in tissue.

umbrella of the PMI share in the promise to significantly decrease the morbidity and mortality of cancer patients.

There are many modalities available for clinical imaging applications, including radiological, magnetic resonance (MR), ultrasound, and optical imaging. However, while many of these technologies are already integral in early diagnosis and disease monitoring, their direct integration into operating room (OR) workflows remains more challenging. Specifically, the bulky machinery and logistics of visualizing MR or radiological imaging agents make their use during surgery difficult. Optical contrast agents, on the other hand, have the potential to be utilized during a surgeon's restricted time frame for resection and can be detected with simple camera systems that enable real-time monitoring of probe signals. However, some of the major challenges facing optical contrast agents include sensitivity, tissue penetration, and the need for a clear pathway for clinical development and approval. Many of these challenges can be addressed with the development of suitable instrumentation and new generations of chemical probes with increased selectivity and in vivo properties. This review will

focus on optical imaging agents designed for intraoperative use to provide real-time feedback to surgeons. We will further refine the scope to discuss chemical tools that target enzymatic and metabolic processes upregulated in tumors and the tumor microenvironment.

Small Molecule and Peptide-Based Optical Contrast Agents

We have chosen to focus this review on chemically tractable imaging agents because there are a number of advantages of using such agents for imaging, including cost of production, stability, and potential to be topically applied to areas of interest. Clinically, IV administration or topical application of optical contrast agents could be used at multiple stages of surgical intervention. With the current optical probes and imaging devices, optical contrast agents can be used for intraoperative decision making in vivo or ex vivo when applied to biopsy or surgical tissues (Figure 1). We speculate that these probes will also eventually become an integral technology for the pre-operative planning and decision-making stage, as dyes with enhanced properties and imaging devices with more sensitive detection methods become available (Figure 1). Before advances in these technologies become available, the advantage of using the same contrast agent for pre-operative and intraoperative decision making could be proven using analogs of optical probes that also contain a PET tracer. In this scenario, the PET tracer would be employed for pre-operative planning while the optical reporter would be used intraoperatively. Beyond their clinical utility, these chemical tools have a broad range of applications in the OR when applied in clinical and basic research applications. Importantly, many devices have been designed for the purpose of visualizing optical contrast agents. Currently, a variety of companies manufacture US Food and Drug Administration (FDA)-approved surgical camera systems, such as the Firefly system in the da Vinci Surgical System (Buchs et al., 2012), the Pinpoint camera (Sherwinter, 2012), the Fluorescence-Assisted Resection and Exploration (FLARE) and Mini-FLARE image-guided surgery systems (Tummers et al., 2015; Verbeek et al., 2013, 2014a, 2014b), the Photo Dynamic Eye (PDE) and PDE-neo (Aoki et al., 2010), the Hyper Eye Medical System (HEMS) (Yoshida et al., 2012), and the IMAGE1 SPIES near-infrared/indocyanine green (NIR/ICG) system (Schols et al., 2013a). Similarly, there are multiple devices for use in basic science applications and in animal models of disease, including the IVIS, Maestro, Fluobeam, MiroSurge, Lab-FLARE Model R1 Open Space Imaging System, and a range of confocal microscopes. While there are a significant number of approved imaging devices, there are no molecularly targeted optical contrast agents that are FDA-approved for clinical use. Thus, all of the current clinical work makes use of non-targeted dyes such as indocyanine green (ICG) (Buchs et al., 2012; Verbeek et al., 2014a; Yoshida et al., 2012), methylene blue (MB) (Tummers et al., 2015; Verbeek et al., 2013), and fluorescein (Gribar and Hamad, 2007; McGinty et al., 2003). However, given the availability of imaging devices, efforts to advance optical contrast agents should be a priority, as new agents can be immediately integrated into surgical workflows as they become clinically available. A summary of the optical probes highlighted in this review and their stage of pre-clinical or clinical development is given in Table 1.

Table 1. List of Probes Highlighted in This Manuscript and Their Pre-clinical or Clinical Status

Name	Pre-Clinical/Clinical Trials	Notes
Tumor Paint (BLZ-100)	Phase I clinical trials for adult skin cancer (completed March 2015), sarcoma, pediatric tumors of the CNS, glioma, and other solid tumors	NCT02097875, NCT02464332, NCT02462629, NCT02234297, NCT02496065
CLR1501 and CLR1502	pre-clinical animal models	(Swanson et al., 2015)
OTL38	Phase II clinical trial for FR α -positive ovarian cancer	NCT02317705
cRGD-ZW800-1	pre-clinical animal models	(Choi et al., 2013)
YC-27	pre-clinical animal models	(Neuman et al., 2015)
PSMA-1-IR800	pre-clinical animal models	(Wang et al., 2014)
Fluorocoxib A	pre-clinical animal models	(Ra et al., 2015; Uddin et al., 2010)
BMV109	pre-clinical animal models	(Segal et al., 2015; Verdoes et al., 2013)
LUM105	Phase I clinical trials for sarcomas, soft tissue sarcomas and breast cancer; feasibility clinical trials for breast cancer and GI cancers of the colon, esophagus, and pancreas	NCT01626066, NCT02438358, NCT02584244
C-PGC probe	pre-clinical animal models	(Weissleder et al., 1999)
MMP-2-sensitive NIRF probe	pre-clinical animal models	(Bremer et al., 2001)
Z-Phe-Arg-HMRG	pre-clinical animal models	(Fujii et al., 2014)
Lipidated probe 3	pre-clinical animal models	(Hu et al., 2014)
6QCNIR	pre-clinical animal models	(Ofori et al., 2015)
RACPP AVB-620	Phase I clinical trial for breast cancer	NCT02391194
C-SNAF	pre-clinical animal models	(Ye et al., 2014)

Considerations for Probe Development

In general, an optical chemical probe consists of three main structural elements: the recognition element, the linker, and the dye (Figure 2A). The recognition element enables targeted delivery of the probe to the cells of interest. Specifically targeting molecular signatures of cancer cells by the recognition element is perhaps the most important component to optimize in the design of an optical probe. The two primary strategies used to induce tumor-specific probe accumulation include (1) optimizing binding affinity for molecular targets that are over-represented in cancer cells or cells associated with a solid tumor (affinity agents) and (2) efficient response to enzymatic signatures found mainly in the cancer cell or tumor-associated cells (substrate and activity-based probes) (Figure 2). These two approaches, affinity-based and enzymatic targeting elements, each have strengths and weaknesses. Recognition elements of affinity-based probes are usually based on the endogenous ligand of an enzyme or receptor that is upregulated in cancerous cells. Alternatively, screening approaches or direct optimization of small molecule ligands can be used to yield probes that have selective affinity for cancer-specific targets. Affinity-based probes largely rely on the accumulation and retention of the dye-containing molecule due to target binding and require removal of the unbound probe to generate contrast. In contrast, enzyme substrates and activity-based probes can be designed to only produce signal or accumulate in the tumor upon processing by a cancer-specific enzyme. This usually involves enzymes that cleave recognition motifs such as proteases, or enzymes that react with the recognition motifs to produce a signal. In both strategies, an important aspect to consider is the localization of the target of interest to the intracellular compartment, extracellular compartment, or specific organelles. Direct studies assessing the challenges and benefits of choosing a target localized to

each of these compartments have yet to be carried out. However, one possible challenge to choosing an intracellular target, or a target localized to a specific organelle, is that these probes must cross the cell membrane to interact with their target of interest. Conversely, a possible challenge posed with extracellular targets is signal attenuation due to diffusion.

In addition to the mechanism of targeting, it is important to optimize the type of reporter dye used and how these reporters are attached to the targeting group. Selection of an optimal dye is an important aspect of probe design. Because small-molecule probes are by their very nature relatively small in size, the attachment of large quenchers and dyes to the core recognition element can dramatically impact the in vivo properties, cell permeability, and biodistribution of the probe. Furthermore, the excitation and emission wavelengths of the dye are key considerations. Near-infrared (NIR) light lies outside the range of tissue autofluorescence and can detect tumors up to 5–10 mm into tissues (Rosenthal et al., 2015a; Schols et al., 2013b; Vahrmeijer et al., 2013). Thus, virtually all optical imaging agents for clinical applications make use of NIR dyes. There are currently many commercially available dyes that cover the range of NIR wavelengths, and agents with increasingly longer wavelengths are proving to be useful for increased signal and reduced background (Antaris et al., 2015). In addition to the excitation and emission wavelengths of the dye, the overall balance of charge-to-hydrophobicity of the chemical structure also plays an important role in the signal-to-background ratio output of the probe (Choi et al., 2013). By employing zwitterionic dyes, it is possible to achieve a well-balanced charge distribution over the surface of the chemical probe, allowing for a more hydrophilic molecule with low nonspecific binding properties (Choi et al., 2013).

Contrast agents can be optimized in the linker region that attaches the dye to the targeting element, as well as in the dye

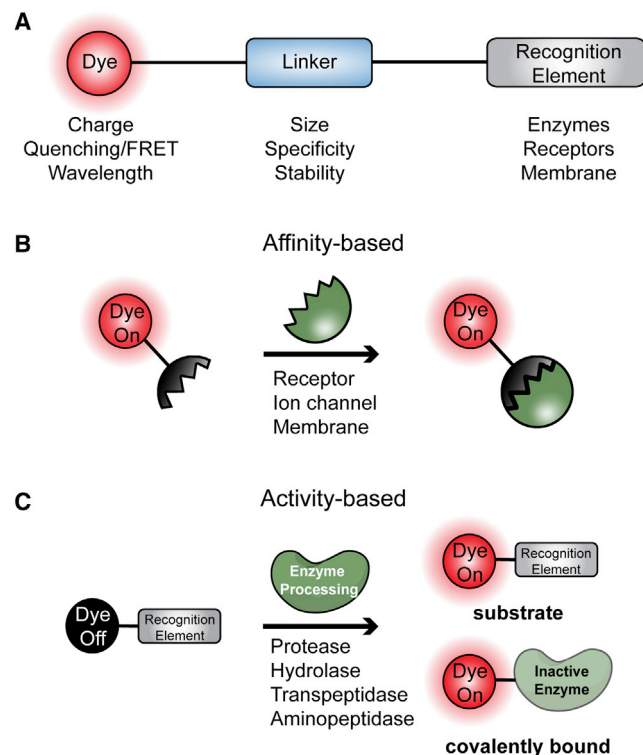


Figure 2. General Considerations for Probe Design

(A) Optical chemical probes consist of three main structural elements: dye, linker, and recognition element.
 (B) General model for affinity-based probes. The probe binds to targets such as receptors, ion channels, and membranes with high affinity.
 (C) General model for activity-based probes. These probes give off a fluorescent signal upon processing by enzymes such as proteases and peptidases.

placement on the probe. Varying the linker (to include PEG or poly amino acids) to control the overall size of the probe influences overall properties of the probe, such as rate of diffusion, cell permeability, and plasma circulation times. Larger probes take advantage of the enhanced permeability and retention effect (EPR), a tendency of larger molecules to accumulate in tumor tissue to a greater extent than in healthy tissue (Matsumura and Maeda, 1986). However, this can come at the price of speed of signal generation, as larger probes tend to penetrate solid tumors at a slower rate than small probes (Blum et al., 2009). Additionally, the linker can be used to change the overall charge of the molecule or to localize it to specific intracellular locations. Finally, the placement of the dye molecule can dramatically impact the imaging properties of the probe, as will be discussed for several of the probes highlighted in this review.

Affinity-Based Probes

Affinity-based probes generally exploit the fact that certain proteins, such as enzymes and cell surface receptors, are upregulated in cancer cells (Figure 3). Furthermore, there are a number of naturally occurring ligands with high intrinsic affinity for cancer targets that can be used as starting points for probe design. In some cases, this can involve the simple attachment of a suitable fluorescent dye to generate the imaging agent.

One example of a promising new affinity-based imaging agent is Tumor Paint (BLZ-100) (Figure 3A). This peptidic probe is derived from chlorotoxin venom (CTX) of the *Leiurus quinquestriatus* scorpion (Lyons et al., 2002; Soroceanu et al., 1998; Veiseh et al., 2007), which binds preferentially to the surface of cancerous glioma cells in the brain (Lyons et al., 2002; Soroceanu et al., 1998). Interestingly, the specific molecular target of CTX is not yet clear, and reports have suggested multiple possible targets, including the glioma-specific chloride ion channel (GCC), a lipid raft-anchored complex that binds to matrix metalloproteinase-2 (MMP-2), membrane type-1 MMP, transmembrane inhibitor of MMP-2 (TIMP2), and CIC-3 chloride ion channel (Deshane et al., 2003; Soroceanu et al., 1998; Veiseh et al., 2007). CTX is a 36 aa peptide that contains four disulfide bonds, causing it to fold into a compact and well-defined structure (Soroceanu et al., 1998; Veiseh et al., 2007). CTX was developed into an optical imaging agent by the Olson group through conjugation with a Cy5.5 NIR fluorophore (Veiseh et al., 2007). Because CTX contains three lysine residues, Cy5.5 conjugation resulted in a mixture of mono-, di-, and tri-labeled probes (Akcan et al., 2011). This posed a regulatory and manufacturing problem for translation to the clinic and required re-engineering of the CTX peptide to replace Lys15 and Lys23 with alanine or arginine (Akcan et al., 2011). Modification of these two residues resulted in specific Lys27 labeling to generate a probe that retained the stability and in vivo half-life properties of the original peptide (Akcan et al., 2011). Interestingly, cyclization of CTX containing all three lysine residues resulted in only a single Cy5.5-labeled species but decreased the in vivo half-life to 11 hr, as compared to the 14 hr half-life of the linear peptidic probe (Akcan et al., 2011). Although originally designed based on its glioma-specific targeting, Tumor Paint has now been used in a variety of cancers, including medulloblastoma, sarcoma, and carcinomas of the prostate, colon, breast, lung, skin, and intestines (Akcan et al., 2011; Veiseh et al., 2007). Importantly, the company Blaze Biosciences (<http://www.blazebioscience.com>), which has a license to the Tumor Paint technology, has one completed and ongoing Phase I clinical trials investigating the use of BLZ-100 in adult skin cancer (completed March 2015), sarcoma, pediatric tumors of the CNS, glioma, and other solid tumors (<http://www.clinicaltrials.gov> identifiers: NCT02097875, NCT02464332, NCT02462629, NCT02234297, NCT02496065).

An alternate approach to generate affinity-based probes uses modified metabolites and biosynthetic molecules that are known to accumulate in tumors. For example, phospholipid ethers (PLEs) were found to accumulate to higher levels in cancer cells compared to normal tissues as early as the 1960s (Snyder et al., 1969; Snyder and Wood, 1969). At the same time, PLE and alkyl-phosphocholine (APC) analogs were shown to exhibit cytostatic and anti-tumor activities (Andreesen et al., 1978). The anti-cancer activity of phospholipid analogs is thought to occur upon insertion into cellular membranes, which disrupts phospholipid and cholesterol metabolism, the function of lipid rafts, and the homeostasis of important signaling pathways such as inositol triphosphate (IP₃) and calcium signaling, the PI3K/Akt pathway, and the MAPK pathway (van Blitterswijk and Verheij, 2013; Weichert et al., 2014). Taking advantage of the tumor-specific uptake of PLEs and APCs, the APC derivative octadecyl phosphocholine has been conjugated with BODIPY (CLR1501) and

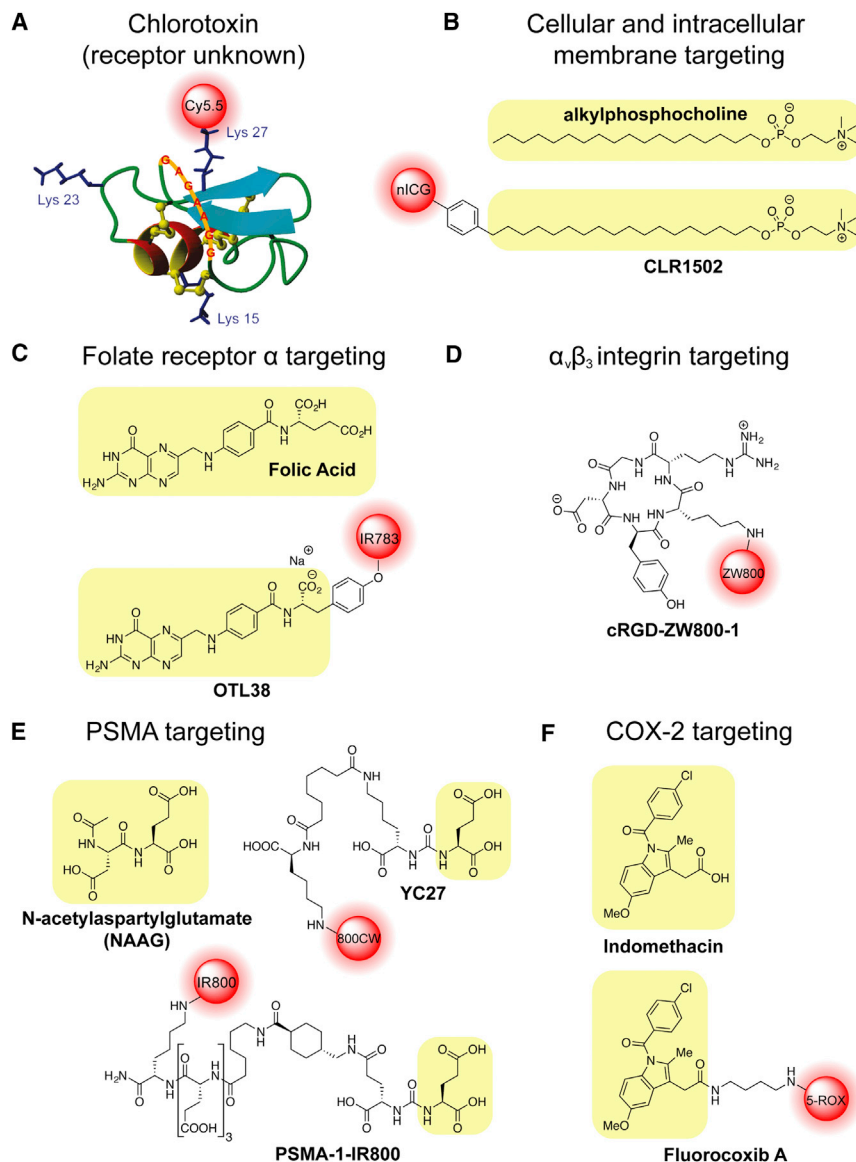


Figure 3. Affinity-Based Probes

(A) Chlorotoxin-based probe BLZ-100. (B) Alkylphosphocholine (APC) structure and APC-derived probe CLR1502. (C) Folic acid structure and folate receptor α -targeting probe OTL38. (D) Cyclic RGD peptides as tumor contrast agents. (E) NAAG structure and PSMA targeting probes YC27 and PSMA-1-IR800. (F) Structure of Indomethacin and the corresponding labeled probe Fluorocoxib (A).

NM404), as well as optical agents (CLR1501 and CLR1502). While no clinical trials have been initiated for the optical imaging agents CLR1501 or CLR1502, CLR1404 was advanced into animal proof-of-concept studies as well as multiple Phase I/II clinical trials by Cellectar Biosciences (<http://cellectarbiosciences.com>) (<http://www.clinicaltrials.gov> identifiers: NCT00582283, NCT00925275, NCT01662284, NCT01516905).

One of the more common strategies for affinity probe design is to exploit cell surface receptors known to be upregulated in cancer. The folate receptor α (FR α) is a promising target for directed diagnosis, imaging, and therapies in cancer treatment. Its high level of overexpression on the surface of many cancer types, including carcinomas of the ovary, breast, lung, kidney, and colon, make it an ideal target for molecularly targeted agents (Parker et al., 2005; Srinivasarao et al., 2015). Studies have reported levels of FR α expression on cancer cells as high as 280,000 receptors per cell (Destito et al., 2007; Saul et al., 2003). Furthermore, in normal tissues, with the exception of cells in the proximal tubule of the kidney and lung, expression of FR α is

new ICG (CLR1502) to generate optical imaging agents (Figure 3B) (Swanson et al., 2015). Significant SAR analyses have also been performed on PLE analogs to optimize their tumor uptake and pharmacokinetic properties, including plasma half-life, biodistribution, and clearance (Pinchuk et al., 2006). Alkyl chain length was found to be important for tumor targeting and clearance, with the increased hydrophilicity of C7 analogs resulting in decreased tumor targeting and rapid clearance (Pinchuk et al., 2006). Increasing the alkyl chain length to 18 carbons afforded increased plasma half-life when compared to C15 analogs (Pinchuk et al., 2006). Derivatization to propanediol analogs resulted in undesirable retention of the probe in hepatocytes, and adding a 2-O-methyl group to the glycerol backbone decreased tumor uptake (Pinchuk et al., 2006). These SAR studies identified the optimal APC analog as the C18 octadecyl phosphocholine, which was made into radiolabeled 18-(*p*-Iodophenyl)octadecylphosphocholine (CLR1404, formerly

confined to the apical surface of cells, rendering it inaccessible to therapeutics or contrast agents due to adherens and tight junctions (O'Shannessy et al., 2012; Parker et al., 2005; Weitman et al., 1992; Yang et al., 2012). These qualities result in high signal-to-noise ratios for agents specifically targeting FR α (Srinivasarao et al., 2015; van Dam et al., 2011; Weitman et al., 1992; Yang et al., 2012). Furthermore, FR α binds folate (vitamin B₉) with high affinity to facilitate its uptake for single-carbon metabolism, a process that is particularly important for amino acid metabolism and nucleotide synthesis (Antony, 1996). To generate optical probes based on this cancer-specific target, Low et al. created the first-generation probe EC17 by conjugating folate to fluorescein isothiocyanate (FITC) (van Dam et al., 2011). EC17 was used for a small human clinical study in patients with ovarian cancer. The trial confirmed that the probe detected FR α overexpression in a subset of these patients and improved clinicians' ability to detect cancer lesions (van Dam

et al., 2011). Further optimization led to a related compound, OTL38, which uses a folate analog, pteric acid-tyrosine conjugated to IR-783 (Figure 3C) (Srinivasarao et al., 2015). On Target Laboratories (<http://www.ontargetlaboratories.com>), the company licensing OTL38, is currently investigating this probe in a Phase II clinical trial for intraoperative imaging in patients with FR α -positive ovarian cancer, with an expected completion date in 2016 (<http://www.clinicaltrials.gov> identifier: NCT02317705).

Alternatively, many types of targeted contrast agents have made use of the high expression of cell-surface adhesion molecules. The integrins make ideal receptors for imaging agents because they are highly expressed on the surface of many types of cancer. In particular, $\alpha_v\beta_3$ integrin can be targeted using short peptides that contain an RGD sequence. Using this approach, Frangioni et al. have made cyclic RGD peptides linked to NIR dyes for optical imaging of tumors (Figure 3D) (Choi et al., 2013). These probes also make use of a novel zwitterionic dye that has improved signal-over-background ratios in vitro and in vivo compared to more highly charged, dipole-like commercial dyes (Choi et al., 2013).

Another cell-surface receptor that has generated significant interest as an imaging target due to its high expression levels in prostate cancer cells is the zinc metalloenzyme prostate-specific membrane antigen (PSMA) or glutamate carboxypeptidase II (Banerjee et al., 2008; Schulke et al., 2003). Prostate cancer is the most prevalent cancer in men (Howlander et al., 2014; U.S. Cancer Statistics Working Group, 2015), with an incidence of 196,038 in the US (Henley et al., 2014), and 138,000 prostatectomies were performed in 2010 alone (Centers for Disease Control and Prevention, 2012). In addition to the high volume of prostatectomy surgeries performed each year, this surgery presents a challenge due to the desire to preserve critical autonomic nerves that run through the prostatic fascia for patients without extracapsular extension (Lepor, 2005). Therefore, optical tools to intraoperatively define whether tumor margins extend beyond the prostate capsule would lead to significant decreases in morbidity and other complications associated with prostatectomy.

Several groups have designed high-affinity optical and radiological probes to target PSMA. Some of the most successful and clinically advanced probes use urea-based scaffolds based on the structures of the high-affinity PSMA inhibitors 2-(phosphomethyl)pentanedioic acid (PMPA) and phosphonic bis-dicarboxylic acid (Kozikowski et al., 2001; Pomper et al., 2002). To generate the optical probe YC-27, the NIR dye IRDye800CW was linked to cysteine-glutamate or lysine-glutamate urea inhibitors, resulting in compounds that retained their high affinity for the PSMA active site and selectively targeted PSMA+ xenografts over PSMA− xenografts in murine and porcine models (Figure 3E) (Banerjee et al., 2011; Chen et al., 2009; Neuman et al., 2015). A dual-modality radionuclide- and optically-labeled probe was created using the lysine-glutamate IRDye800CW urea labeled with radioactive ^{111}In (Banerjee et al., 2011). This dual-modality probe retained similar potency to that of the singly labeled NIRF and radionuclide probes and displayed rapid and specific labeling in mice with PSMA+ xenografts when visualized using sequential Single Photon Emission Computed Tomography and X-ray Computed Tomography (SPECT-CT) and optical

imaging (Banerjee et al., 2011). Furthermore, a fluorine-18-labeled molecule, *N*-[*N*-[(*S*)-1,3-Dicarboxypropyl]carbamoyl]-4-[^{18}F]fluorobenzyl-L-cysteine (^{18}F DCFBC), has been used in human studies and early-phase clinical trials to detect primary and metastatic prostate cancer (Cho et al., 2012; Mease et al., 2008; Rowe et al., 2015a, 2015b) (<http://www.clinicaltrials.gov> identifiers: NCT01815515, NCT02190279, NCT01417182, NCT01496157).

An alternative approach created a high-affinity ligand, PSMA-1, by attaching a linker element containing three D-glutamic acid residues to the parent cysteine-glutamate urea targeting element (Wang et al., 2014). The increased negative charge of the probe from the glutamic acid residues decreased background signal, while the use of D-amino acids increased the in vivo stability of the molecule (Huang et al., 2014; Kozikowski et al., 2001; Wang et al., 2014). Furthermore, the linker increased binding affinity 4.3-fold compared to the original Cys-Glu parent molecule (Wang et al., 2014). Interestingly, the binding affinity of PSMA-1 conjugated to the IR800 dye further improved affinity, while the use of the Cy5.5 fluorophore did not, highlighting the importance of dye selection in probe design (Figure 3E) (Wang et al., 2014). These PSMA-directed optical probes have yet to enter into human clinical trials, but numerous clinical trials utilizing PSMA-directed contrast agents for diagnostic PET or MR imaging highlight the potential for these types of agents in clinical practice (Barrett et al., 2013; Cho et al., 2012; Mease et al., 2008; Rowe et al., 2015a, 2015b) (<http://www.clinicaltrials.gov> identifiers: NCT02190279, NCT02488070, NCT02048150, NCT01496157, NCT02420977, NCT01815515, NCT02282137, NCT01173146, NCT00992745, NCT00712829).

In another example of a probe based on a high-affinity small-molecule inhibitor, Marnett and coworkers have engineered fluorescently labeled probes that target the enzyme cyclooxygenase-2 (COX-2). This enzyme is not normally expressed in healthy tissues but has high expression levels in sites of inflammation and neoplasia (Eberhart et al., 1994). Thus, it is an ideal imaging biomarker that has been shown to be upregulated in colon, prostate, breast, pancreatic, lung, and skin cancers (Sobolewski et al., 2010). Initial design efforts for COX-2 probes involved significant SAR studies to identify selective COX-2 inhibitors as well as an optimal dye for coupling to the parent inhibitor molecule (Uddin et al., 2010, 2013). The authors identified the nonselective COX inhibitor indomethacin as the best affinity scaffold over other COX inhibitors such as celecoxib and nonsteroidal anti-inflammatory drugs (NSAIDs) (Uddin et al., 2013). Furthermore, fluorescent conjugation to indomethacin scaffolds, as well as optimizing the length and electronic properties of the linker, could confer selectivity in inhibition to COX-2. For instance, the red fluorophore 5-ROX conferred COX-2 selectivity but was only potent when the ethylenediamide linker was increased from two to four carbons, illustrating the significant effects that the chemical properties of the dyes and linkers have on overall probe performance (Uddin et al., 2013). This COX-2 probe, termed fluorocoxib A (Figure 3F), has been used to image colon cancer polyps (Uddin et al., 2010) as well as non-melanoma skin cancer using non-invasive detection methods (Ra et al., 2015); however, use of the 5-ROX fluorophore (λ_{ex} = 580 nm, λ_{em} = 605 nm) may limit its clinical translatability to human patients.

In addition to these affinity-based probes that are currently advancing to, or are already in, clinical trials, there are a number of commercially available optical imaging tools for basic cancer research and preclinical animal studies. Perkin Elmer markets a folate receptor-specific probe, the FolateRSense 680; the IntegrSense probe, a non-peptidic small molecule designed as an integrin $\alpha_v\beta_3$ antagonist; BombesinRSense 680, a 7-aa NIR-labeled peptide analog; OsteoSense, a bisphosphonate probe targeting bone growth and resorption; Transferrin-Vivo probe, which utilizes transferrins to target transferrin receptors; and the XenoLight RediJet probe, which targets COX-2 using a high-affinity inhibitor (<http://www.perkinelmer.com/Catalog/Category/ID/Targeted>). All of these probes make use of ligands with high affinity for a given target to generate optical imaging probes that follow distinct cell types or enzymatic targets associated with disease.

Finally, though this review focuses mainly on chemically tractable optical probes, there are a number of clinically advanced optical probes composed of monoclonal antibodies (mAbs) conjugated to fluorescent reporters. These antibody-based probes utilize the same targeting principle as the recognition elements of affinity-based probes; that is, they exploit targets overexpressed on tumor cells in comparison to healthy tissue. Many of these clinically advanced probes exploit mAbs and fluorescent tags that, as separate entities, have already been approved for clinical use. For example, approved mAbs specific to epidermal growth factor receptor (EGFR), the chimeric (human/mouse) mAb cetuximab and the fully human mAb panitumumab, have been conjugated to IRDye800. Both mAb-based probes are currently in Phase I clinical trials for use in head and neck squamous cell carcinoma (HNSCC) (Rosenthal et al., 2015c) (<http://www.clinicaltrials.gov> identifiers: NCT01987375, NCT02415881). In addition to EGFR-specific mAbs, the mAb bevacizumab specific to vascular endothelial growth factor A (VEGF-A) has been conjugated to IRDye800CW for use in tumor resection. This mAb-based probe has been used in Phase I and II clinical trials to aid in the removal tumors in breast cancer (<http://www.clinicaltrials.gov> identifiers: NCT01508572, NCT02583568) and is also being investigated in a Phase I clinical trial as an endoscopic contrast agent to detect adenomas in patients with familial adenomatous polyposis (<http://www.clinicaltrials.gov> identifier: NCT02113202). More detailed discussion of these antibody-based probes and their design strategies can be found in the reviews by Nguyen and Tsien (2013) and Warram et al. (2014).

There are a number of advantages and disadvantages to the affinity-based approach. Because many protein biomarkers of cancer already bind to specific ligands, probes can be built by simply attaching reporter groups to an existing high-affinity ligand. Optimization of the linker and dye can further improve affinity and in vivo properties of these tools. A further advantage is that the reporter tag can be readily replaced with other imaging tracers (i.e., for PET or MR imaging) or with therapeutic payloads, thus utilizing the specificity of the probe to not only report on the site of tumor cells but also selectively deliver therapeutic agents. However, affinity reagents are limited by the binding specificity of the probe and expression levels of the target in the tumor. Target expression levels can differ between different patients with the same type of tumor (van Dam et al.,

2011), as well as among different tumor cells within the same patient due to tumor heterogeneity. This highlights the importance of pre-operative biopsies to ensure that the target of interest is expressed before intraoperative use, as well as the need to continue to improve the dyes and technologies in optical contrast agents to allow noninvasive pre-operative planning and diagnostics. Until tools that allow full-body scanning with these probes becomes a reality, dual optical/PET probes could be used for this purpose. Finally, the primary disadvantage of these affinity-based probes is that imaging contrast is only generated by removal of unbound agents and is therefore controlled by the diffusion rate of the probe and biological clearance in vivo. This generally prevents affinity agents from being used on short timescales or for local delivery in topical application.

Substrate and Activity-Based Probes

Activity-based probes (ABPs) and enzyme substrates are molecules that measure the activity of an enzymatic target. ABP and substrate probes exploit the catalytic activity of a target to generate an imaging signal. The main difference between ABPs and substrate probes is that substrates are processed and released by the target enzyme while ABPs remain covalently bound to the active site. Like affinity probes, ABPs and substrates are typically designed to target specific enzymatic activities associated with a particular disease tissue. To provide specific contrast, they make use of a change in fluorescent signal upon processing or binding to the enzyme target. This includes a quenching mechanism in which only the product is fluorescent or a fluorescence energy transfer (FRET) mechanism where fluorescent signals change wavelength upon processing. This ability to report signal only when acted on by a target enzyme allows rapid imaging, as there is no need to remove the unbound agent. A number of strategies have been developed to take advantage of changes in enzyme activities in cancer to generate signal contrast (Figures 4 and 5).

Many groups have developed protease-based ABPs and substrate probes, focusing efforts on specific proteases upregulated in cancers. Specifically, matrix metalloproteinases (MMPs) and serine proteases degrade the extracellular matrix (ECM), allowing the cancer to invade surrounding tissues (Bromme, 2011; Mohamed and Sloane, 2006). Additionally, cysteine cathepsins alter the tumor microenvironment by directly cleaving components of the ECM, such as laminin and fibronectin, as well as adhesion proteins and other proteases, allowing cancer cells to metastasize (Bromme, 2011; Mohamed and Sloane, 2006). They are also highly expressed in activated macrophages, making them ideal markers of both cancer and inflammation. Furthermore, metabolic acidosis in the tumor microenvironment caused by lactic acid buildup from glycolysis induces the activation of extracellular cysteine cathepsins that are released by both tumor and tumor-associated cells such as macrophages (Bromme, 2011; Mohamed and Sloane, 2006). Due to the high levels of cysteine cathepsins in tumors, a number of covalent ABPs and substrates for this class of proteases have been developed.

Fluorescent ABPs that covalently modify cysteine cathepsins were first described over a decade ago and made use of reactive electrophiles such as epoxides, diazomethyl ketones (DMKs),

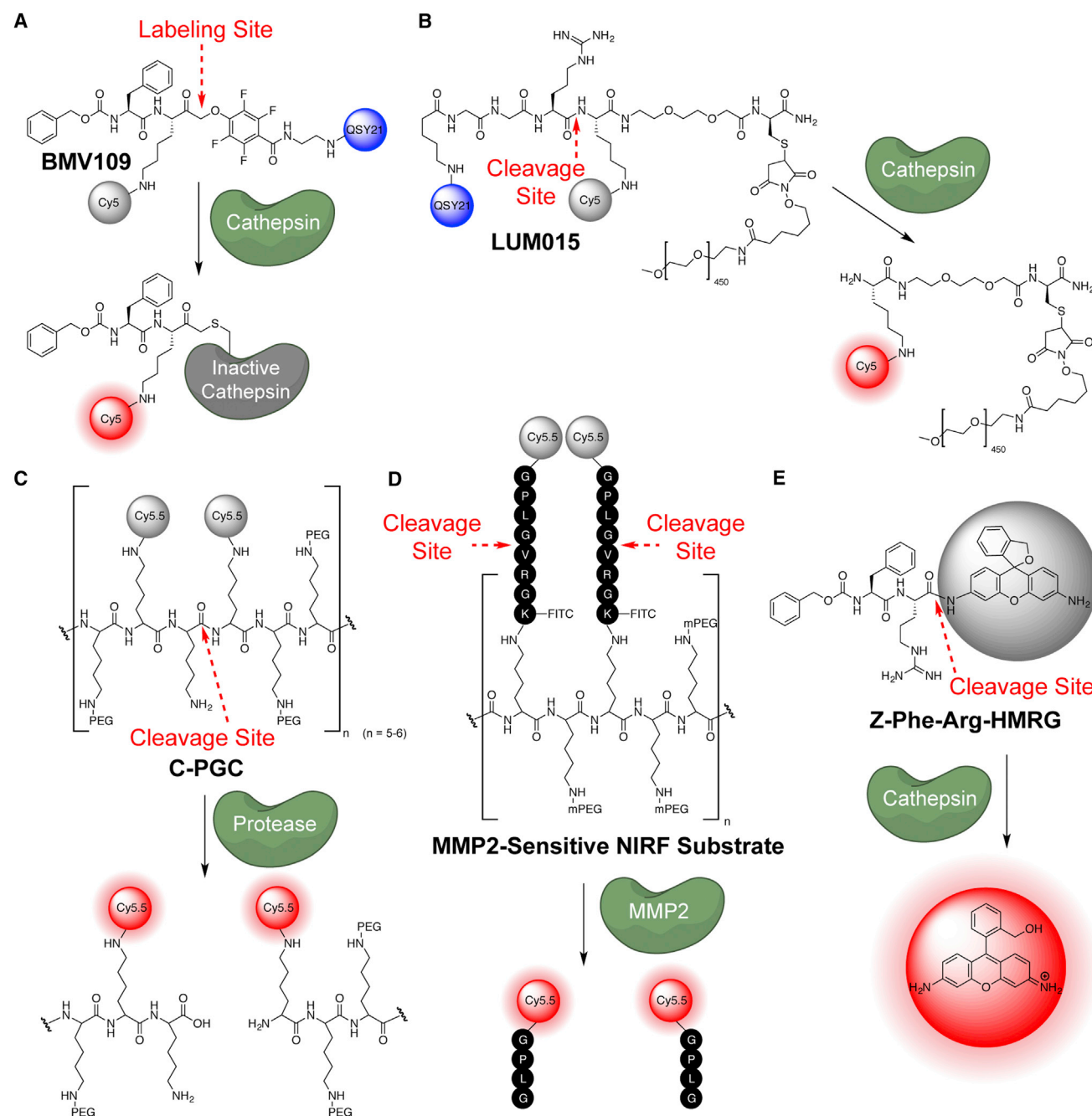


Figure 4. Substrate and Activity-Based Probes

(A) The cathepsin-targeted covalent probe BMV109.
(B) The cathepsin-targeted substrate LUM015.
(C) The polymer-based protease substrate probe C-PGC.
(D) A polymer MMP-2-sensitive NIRF substrate.
(E) The turn-on fluorophore-based probe for cathepsins, Z-Phe-Arg-HMRG.

and acyloxymethyl ketones (AOMKs) (Powers et al., 2002). However, all of the original cathepsin ABPs contained a fluorophore that was not quenched, and, therefore, like the affinity probes, the unbound probe had to be removed to obtain image contrast. A significant improvement came in the form of probes containing a quenching group on the AOMK electrophile that is lost upon

covalent target modification (Blum et al., 2005, 2007). Further refinement of the electrophile to 2,3,5,6-tetrafluoro phenoxy-methyl ketone (PMK) resulted in probes with greatly improved in vivo stability. One such PMK probe, BMV109, is a fluorescently quenched pan-cathepsin probe that is effective at labeling tumors in mouse models of cancer (Figure 4A) (Verdoes et al.,

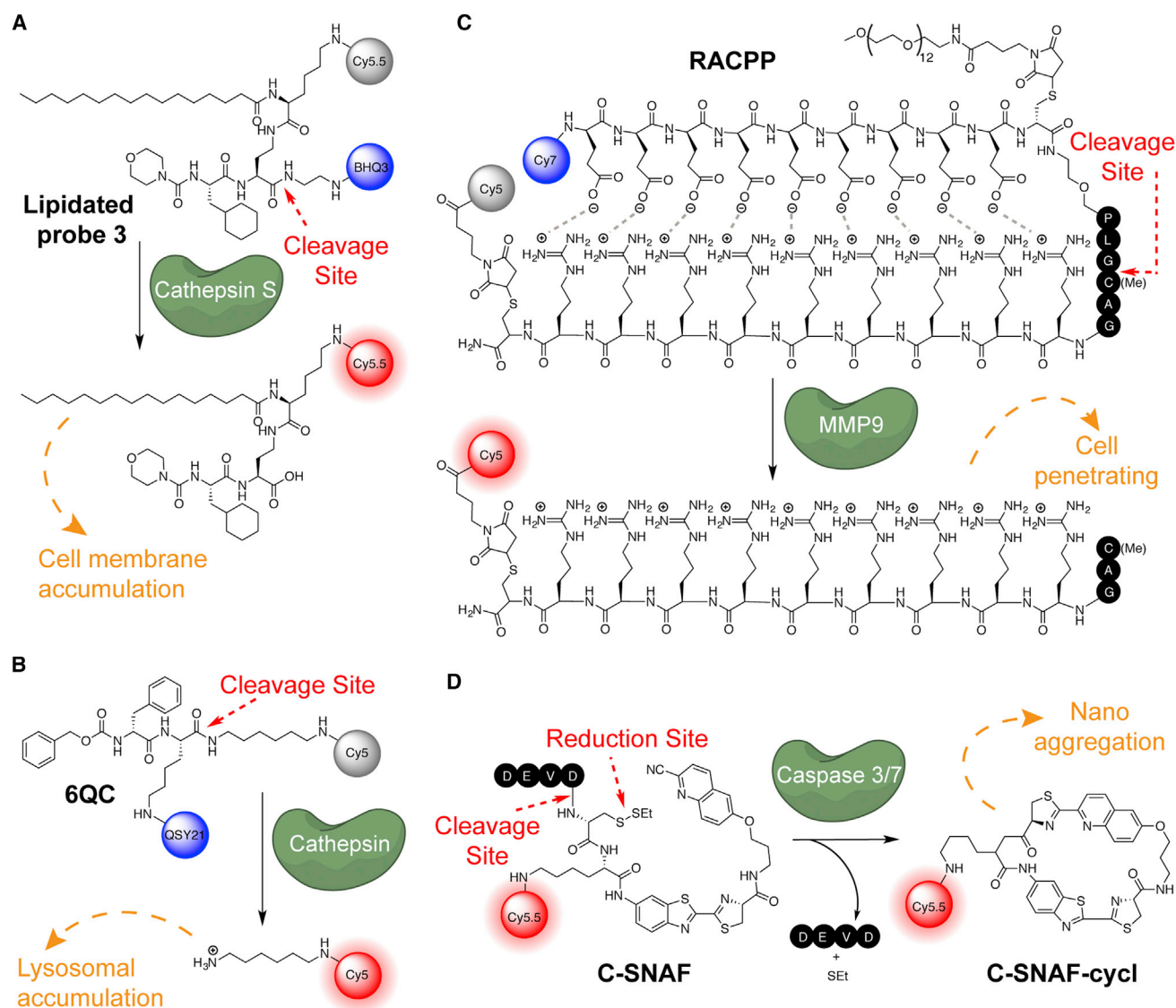


Figure 5. Substrate and Activity-Based Probe Structures

(A) Cathepsin-targeted lipidated probe 3.
(B) The cathepsin-targeting probe, 6QCNIIR.
(C) The cell-penetrating probe, RACPP.
(D) Nano-aggregating probe for caspases, C-SNAF.

2013). Cathepsins recognize the Cbz-Phe-Lys peptide sequence of the probe backbone, and upon cleavage the enzyme is inactivated by the PMK electrophile (Edgington et al., 2013). Because the probe covalently labels target proteases, selectivity of the probe can be directly assessed using mass spectrometry-based approaches. Once the selectivity of the probe is known, SDS-PAGE gel electrophoresis of labeled tissues serves as a direct fluorescent readout to determine which cathepsins have upregulated enzymatic activity in a given tumor tissue. In addition, the covalent nature of the interaction increases signal durability, and the overall small size of the probe provides rapid signal generation. BMV109 has been used for noninvasive optical imaging in animal models of breast and colon cancer and can topically label tissues (Segal et al., 2015). The general peptide

scaffold of the ABP can also be modified to generate non-peptidic analogs with a high degree of selectivity for individual cathepsins, such as cathepsin S-specific probes (Oresic Bender et al., 2015; Verdoes et al., 2012). Furthermore, earlier generations of the covalent cathepsin probes have been used for topical labeling of brain cancer cells (Cutter et al., 2012), suggesting that these small-molecule turn-on probes could be used for direct visualization during surgical intervention.

While covalently labeling proteases has the advantage of localizing the probe to the site of proteolysis and thereby attenuating probe clearance, the disadvantage of enzyme inactivation is that it does not allow signal amplification that results from continued processing events after release of the probe. To generate signal amplification, substrate probes have been

designed that do not covalently inactivate the protease of interest, but are instead cleaved to produce a fluorescent product. Indeed, many groups have shown the utility of employing substrate probes to image protease activity. One of the earliest fluorescently quenched imaging substrates for proteases was generated by conjugating a NIR dye to a poly-L-lysine backbone modified with additional mPEG side chains (Figure 4C) (Weissleder et al., 1999). This probe localizes to the lysosome, where lysosomal proteolytic systems (which include cysteine proteases, serine proteases, and other hydrolases) enzymatically cleave the probe at Lys-Lys junctions. The NIR Cy5.5 dyes are endogenously quenched due to proximity when conjugated to the poly-L-lysine backbone but produce a signal as they are released from the polymer (Weissleder et al., 1999). Using this poly-lysine probe as a foundation, additional classes of imaging probes were designed to target MMPs by inserting a peptide substrate (GPLG*VRGK(FITC)) between the poly-Lys backbone and the fluorophore (Figure 4D) (Bremer et al., 2001). By conjugating many fluorophore/peptide reporter groups to each probe, sites of upregulated MMPs can be visualized with high sensitivity. These probe scaffolds were subsequently developed into the ProSense and MMPsense probes that are commercially available from Perkin Elmer (<http://www.perkinelmer.com/Catalog/Category/ID/Activatable>).

In another example of protease substrate probes, Turk et al. designed probes based on the structures of highly selective inhibitors by converting them into substrates of specific cathepsin proteases (Hu et al., 2014; Watzke et al., 2008). Using this “reverse-design” approach, they engineered a substrate-based probe that is highly selective for cathepsin S (Link and Zipfel, 2006). The probe was designed from a non-peptidic, high-affinity inhibitor to control selectivity and benefit from the optimized in vivo properties of the drug scaffold. These substrate probes produce a high signal in transplanted tumors in a short time frame, generating signal as soon as 30 min after probe injection (Hu et al., 2014).

One of the major issues with using substrate probes is that the product of the cleavage reaction is usually a small-molecule fragment with the potential to diffuse away from the site of interest. This is particularly apparent with larger polymer-based probes, where the intact non-fluorescent imaging agent is slow to enter tissues and the cleavage products have more rapid diffusion out of the tumor, resulting in slow turn-on and rapid signal loss (Blum et al., 2009; Edgington et al., 2009). To address this issue, several groups have used chemical appendages such as lipids (Hu et al., 2014) and PEG linkers (Lee et al., 2014) to increase signal retention. In a recent example, Turk et al. optimized the substrate-based cathepsin probe from Watzke et al. mentioned above by adding a palmitoyl lipid chain to promote cell membrane localization (termed lipidated probe 3; Figure 5A) (Hu et al., 2014). In a 4T1 grafted tumor mouse model, the cleavable substrate functionalized with palmitic acid on the reporter end achieved a signal twice as bright as the previous non-lipidated probe after 24 hr, with continued signal accumulation for 8 days post-injection (Hu et al., 2014). The authors attribute the long-term retention of the probe as well as the high signal-to-noise ratio to the lipid attachment and hypothesize that the lipid functionalization promotes binding of the probe to albumin in the blood, increasing circulation times. Additionally, the authors

speculate that lipidation localizes the probe predominantly to the cell surface, allowing the signal to accumulate after cleavage and blocking diffusion out of the cell. The rapid signal production of these agents compared to the original ProSense polymer probes is likely due to the fact that the relatively small size of the reverse-designed probes allows rapid diffusion into the tumor tissues.

There are examples of successful molecular probes that are currently in clinical trials that make use of a large polymer attached to the cleaved substrate. LUM015 (Lee et al., 2014), a chemical probe developed by Lumicell (<http://lumicell.com>), has shown promising results in mice and dogs (Lee et al., 2014). It is a fluorescently quenched substrate probe consisting of a PEGylated (average 450, MW = 22 kDa) NIR Cy5 fluorophore attached to a QSY21 quencher (Figure 4B). A pan-cathepsin Gly-Gly-Arg recognition sequence was chosen to allow its use in imaging a variety of cancers. LUM015 has been tested in mouse models that were genetically engineered to develop sarcomas (Kirsch et al., 2007), as well as in xenograft (MMTV) breast cancer mouse models. When injected 6–24 hr prior to surgery, no adverse effects were seen (even at 25× normal dose) in mice, and the Lumicell group were able to achieve 12.8× (sarcoma) and 8.3× (xenograft) signal-to-noise ratios in their mouse models. Additionally, Lumicell has used their probe in a preclinical study of canines with naturally occurring cancers. LUM015 showed no related adverse effects in these 12 canines, and intra-operative imaging of 49 tissue samples showed 100% correlation with the pathology results confirming cancer (Lee et al., 2014). LUM015 is currently in Phase 1 clinical trials for soft-tissue sarcomas and breast cancer, as well as feasibility clinical trials for breast cancer and gastrointestinal cancers of the colon, esophagus, and pancreas (<http://www.clinicaltrials.gov> identifiers: NCT01626066, NCT02438358, NCT02584244).

While some probes take advantage of the benefits of large probe size in their design, this strategy may increase background in undesired tissues as well as slow uptake into tumors. To address the problem of rapid diffusion of small-molecule substrate probes while avoiding major modifications to the probe scaffold, one can position the fluorescent reporter to exploit the acidic environment of the lysosome. Since cleavage of the nascent amide bond by a protease target yields a fragment containing a free amine group, placement of the fluorophore on this part of the probe results in a reporter fragment that can be protonated and therefore trapped in the lysosome. This latent lysosomotropic effect (LLE) has been used to design quenched cathepsin protease probes that produce fluorescent fragments with long half-lives after cleavage. One such probe, 6QC NIR (Figure 5B), produced rapid fluorescent signals in cancers of the lung, breast, and colon and was compatible with the FDA-approved da Vinci imaging system equipped with the Firefly detection system (Ofori et al., 2015). Therefore, this class of probes could be readily advanced into clinical studies.

A similar approach to address the problem of signal loss by diffusion is to design probes that expose a cell-penetrating peptide upon cleavage. Therefore, processing by a protease exposes a peptide sequence carrying a reporter dye that rapidly enters cells and becomes trapped. Tsien and Nguyen et al. have designed a ratiometric activatable cell-penetrating peptide

(RACPP) that enters cells and is retained intracellularly upon cleavage by proteases associated with the tumor microenvironment (Savariar et al., 2013). The probe contains an ionic zipper comprised of the cell-penetrating poly-D-arginine sequence modified with a Cy5 dye as well as a stretch of poly-D-glutamates linked to a Cy7 dye (Figure 5C). These two peptides “stick” to each other due to ionic charge interactions and prevent cell penetration by the poly-Arg cell-penetrating peptide. By linking the two peptides with a protease cleavage site, the probe only produces a cell-permeant dye fragment when cleaved by the protease to release the poly-Glu sequence (Savariar et al., 2013). This strategy has been used to generate thrombin-, MMP-, and elastase-specific probes (Jiang et al., 2004; Olson et al., 2009; Savariar et al., 2013; Whitney et al., 2010, 2013). Furthermore, FRET interaction of the two dyes decreases background signal from incomplete quenching and increases overall specific signal by measuring a ratio of one dye fluorescence to the other. This eliminates biases in the overall signal strength that result from differential accumulation of the probe, which can dramatically alter overall signal strength when using quenched probes. Sponsored by Avelas Biosciences (<http://www.avelasbio.com/>), the RACPP probe AVB-620 is currently in Phase 1 clinical trials for surgical removal of breast cancer (<http://www.clinicaltrials.gov> identifier: NCT02391194).

As an alternative to the quenched and FRET-based probes described so far, some probes contain a caged fluorophore, which generates a fluorescent signal upon processing by the target enzyme. Acetylated hydroxymethyl rhodamine green (Ac-HMRG) is a closed spirocyclic, non-fluorescent molecule. However, the non-acetylated HMRG exists as an open molecule that is highly fluorescent. Urano et al. have functionalized this fluorophore with various substrate moieties to produce a fluorescent signal when removed by a target enzyme. Targets include leucine aminopeptidase (Leu-HMRG) (Sakabe et al., 2013), fibroblast activation protein (Ac-GlyPro-HMRG) (Sakabe et al., 2013), β -galactosidase (β Gal-HMRG) (Kamiya et al., 2011), γ -glutamyl-transferase (gGlu-HMRG) (Urano et al., 2011), and, most recently, cathepsins (ZFR-HMRG) (Figure 4E) (Fujii et al., 2014). This type of turn-on dye has great promise, as it is relatively small and does not require additional large quencher groups. Recent work has demonstrated the feasibility of such turn-on dyes in clinically relevant human samples from breast-conserving surgery. In freshly excised human breast specimens, application of the gGlu-HMRG probe for 5 min allowed discrimination of tumors as small as 1 mm in size from normal mammary tissues with 92% sensitivity and 94% specificity (Ueo et al., 2015). The HMRG fluorophore has an emission wavelength of 521 nm, which may limit its use in vivo due to high background and low penetrance of light at this wavelength. It is likely that further design will lead to turn-on dyes with higher wavelength emissions in the NIR range.

Another unique mechanism for generation of turn-on optical probes makes use of an enzyme-induced chemical reaction leading to microaggregate formation that promotes retention of the fluorescent signals inside target cells. Rao et al. designed a chemical probe fitted with an NIR dye that forms microaggregates under specific tumor microenvironmental conditions. The probe contains a cleavage site for caspase-3 and -7 and an additional disulfide-linked thioethyl group. In the reducing tumor

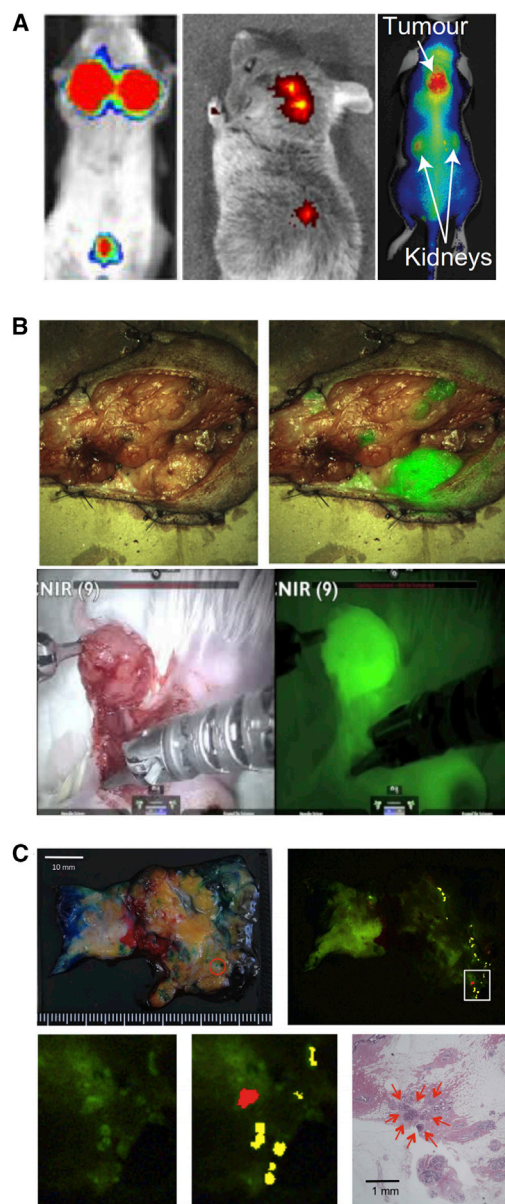
microenvironment with upregulated caspases, the chemical probe C-SNAF undergoes an intramolecular cyclization to produce C-CNAF-cycl that then self-assembles into aggregates in situ (Figure 5D) (Ye et al., 2014). This aggregation can be attributed to the π stacking of C-SNAF-cycl and requires the macrocyclization event. With this probe, Rao et al. were able to non-invasively image apoptosis in HeLa tumor-bearing mice that had undergone three rounds of doxorubicin chemotherapy. Activation by this two-step bio-orthogonal mechanism improves specificity, and its adaptation to target cancer-specific enzymes could be used to improve signal-to-noise signal in cancer imaging.

Outlook and Future Directions for Optical Chemical Probes

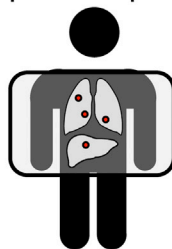
Despite a growing body of basic research and promising evidence gathered in early-phase clinical trials supporting the utility of optical contrast agents for surgical guidance (Figure 6), significant hurdles remain before molecularly targeted contrast agents are approved by the FDA and included in standard surgical workflows. Currently, the only FDA-approved optical imaging agents include ICG, MB, and fluorescein. Even though none of these agents provide any kind of directed targeting, they are finding widespread use in various aspects of surgical care, confirming the clinical value of optical guidance.

An important challenge in translating the chemical tools from academic research laboratories into the clinic involves the regulation and approval process from the FDA. Currently, there is no defined pathway for approval of optical contrast agents, which fall in between categories designated for microdose (defined as less than 100 μ g total dose) imaging agents and therapeutics. At the moment, the doses necessary for optical agents to achieve adequate signal are above the microdose level, yet the agents are only administered once before surgery (Rosenthal et al., 2015b). Additionally, there are regulatory and approval challenges associated with the design of human clinical trials for these agents, as the definition of desired outcomes for Phase II or III clinical trials are not clear. Specifically, randomization would pose a challenge, as the surgeon could not be blinded to the arm of the study in which each patient was placed. Furthermore, there are many confounding factors in assessing the success of surgery or improving overall survival of a given patient or disease, making it difficult to assess efficacy of the contrast agent alone. Given the significant unmet clinical need for surgical guidance tools coupled with the obvious value of improved visual contrast for many aspects of surgery, we speculate that a unique pathway will be required that straddles the requirements for microdose compounds and therapeutics. In much the same way that the FDA is now being called upon to work with scientists to develop pathways for the efficient regulation of new genomic technologies (Collins and Varmus, 2015), we similarly hope it will define a path for optical contrast agents to move into the clinic in a way that preserves efficiency but ensures efficacy and safety.

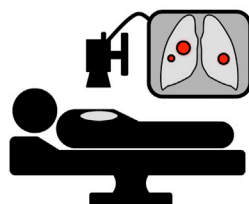
One potential alternate route for rapid generation of human clinical data is to make dual optical/PET agents, or analogs of optical probes functionalized with PET radiolabels in place of the fluorescent dyes. This strategy would allow them to first enter into clinical trials and gain FDA approval using microdosing,



Diagnostics and pre-operative planning



In vivo surgical guidance



Ex vivo surgical guidance

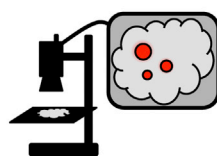


Figure 6. Examples of Optical Chemical Probes Being Used in Animal Models for Non-invasive Cancer Diagnostics, In Vivo, and Ex Vivo Surgical Guidance

(A) Left: 6QC (20 nmol IV), imaged after 4 hr in 4T1 breast cancer mouse model. Middle: Folate-Dylight680 (10 nmol IV), imaged after 4 hr in FR-expressing L1210A tumor mouse model. Right: C-SNAF (5 nmol IV), imaged after 1 hr in 3 \times DOX-treated tumor-bearing mice.

(B) Top: CTX:Cy5.5. Image shows white light and white light with NIR overlay on a canine tumor tissue sample. Bottom: 6QCNIR injected 6 hr prior to surgery. Image shows white light on the left and fluorescence using the da Vinci surgical system on the right.

(C) gGlu-HMRG (3 ml of 5 μ M in 0.5% v/v DMSO in RPMI1640) topically applied to patient specimen diagnosed with invasive ductal carcinoma (papillotubular) and imaged after 5 min. Red and yellow coloring indicate fluorescence-positive areas. Red arrows indicate areas of invasive ductal carcinoma.

As a representative example, we consider the cost-benefit analysis of re-excision surgeries in the treatment of breast cancer using breast conservation surgery, or lumpectomy procedures. The percentage of patients that are post-operatively identified to have positive surgical margins requiring repeated surgeries falls in a wide range, between 17.7% and 72% (Brown et al., 2013; Huston et al., 2006; Jacobs, 2008; Mendez et al., 2006). The costs of re-excision surgeries are high, with the total annual cost for repeated lumpectomy procedures reported by one hospital to be \$273,800, or \$4,721 per patient (Arora et al., 2015). Furthermore, reimbursement rates for re-excision surgeries are low and are not always fully covered by insurance companies, with the same hospital reporting institutional losses from Medicare reimbursements of \$540 per re-excision surgery (Arora et al., 2015). Re-excision surgery represents a high cost burden on the healthcare system and highlights the potential for optical contrast agents to decrease overall healthcare costs while simultaneously improving patient outcomes.

which provides adequate contrast for PET modalities, and to leverage the microdosing regulatory procedures of PET contrast agents to pave the way for an optical imaging analog to follow (Rosenthal et al., 2015b). Ultimately, however, these agents will have to move through alternate routes to become FDA-approved at doses that are adequate for optical-only imaging applications.

Beyond FDA approval, the cost of the agents compared to the potential for reimbursement from insurance companies poses another challenge to the adoption of optical contrast agents into surgical workflows. While it remains unclear what the cost will be to implement optical contrast agents, and costs may also be somewhat probe- and application-dependent, an improvement in patient outcomes coupled with a decrease in re-excision surgery argues for the reimbursement of these tools.

precision imaging is a promising interventional technology that complements the advances of biomedical and clinical medicine in the diagnosis and treatment of human cancers. Many of the chemical probes detailed here have the potential to become clinically available in the next 5–10 years and decrease morbidity associated with surgical procedures. Regardless of the time and cost required to develop such contrast agents, current success with both pre-clinical and clinically applied optical probes suggests that this technology will have a significant positive impact in the quality of healthcare in the future.

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