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Reviews

# Activity-Based Diagnostics: Recent Advances in the Development of Probes for Use with Diverse Detection Modalities

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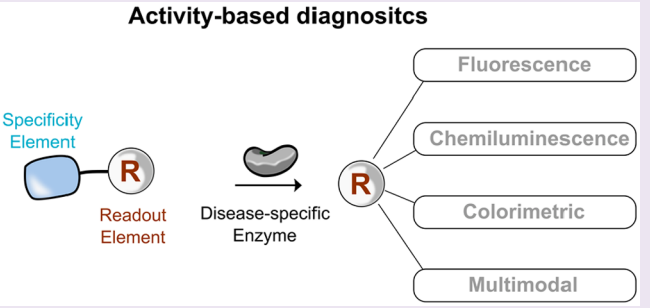
**ABSTRACT:** Abnormal enzyme expression and activity is a hallmark of many diseases. Activity-based diagnostics are a class of chemical probes that aim to leverage this dysregulated metabolic signature to produce a detectable signal specific to diseased tissue. In this Review, we highlight recent methodologies employed in activity-based diagnostics that provide exquisite signal sensitivity and specificity in complex biological systems for multiple disease states. We divide these examples based upon their unique signal readout modalities and highlight those that have advanced into clinical trials.






Diagnostics play crucial roles in modern medicine. A fast and accurate diagnostic can inform treatment regimens and be utilized to measure therapeutic response.[1,2] An ideal diagnostic balances turnaround time, need for specialized instrumentation and trained personnel, and assay sensitivity and specificity. The current gold standard of diagnostics varies based on a variety of factors but is primarily focused on the clinical indication and the human, monetary, and instrumentation resources available.[3] For example, a suspected malaria patient in the western world will likely have their blood analyzed for parasite DNA via RT-PCR due to the high sensitivity and specificity of the assay; however, RT-PCR requires expensive reagents, dedicated instrumentation, and trained personnel to run and analyze the diagnostic assay.[4] A patient in a less resource-rich area, such as sub-Saharan Africa, will likely be tested with less sensitive methods (e.g., microscopy or lateral flow assay) that are significantly less expensive.[5] As such, when developing novel diagnostic assays, one must keep in mind the needs of the patient and the resources afforded in the local community.

Common strategies for diagnostics include pathogen culture, antibody-based antigen detection, DNA and RNA PCR amplification, and noninvasive imaging. Each of these strategies has their own strengths and weaknesses when considering the speed, cost, instrumentation, specificity, and sensitivity of the diagnostics. Historically, culturing has been a critical method for the detection of a variety of pathogens.[6] A prominent example is patients presenting with urinary tract infections (UTIs). These patients will often be diagnosed via a urine culture to identify the infectious microorganism.[7] While microbial culture is a powerful tool to help clinicians diagnose infections, it is severely limited by the time required (typically 24 h) and the difficulty in establishing culture conditions that allow all possible pathogens to grow *ex vivo*. In fact, it is estimated that 50% of bacteria in the mouth alone cannot be cultured,[8] and approximately 30% of all surgical site infections produce culture-negative results.[9] A striking example comes from the bacteria responsible for syphilis, *Treponema pallidum*, which despite infecting tens of millions per year, took over a century before a suitable culturing method was developed.[10]

Antibody-based antigen detection is a powerful tool used for clinical diagnostics and is estimated to reach a $40 billion market cap by 2027.[11] This category encompasses techniques such as ELISA, immunoassays, and immunohistopathology. Flow cytometry is a rapid and relatively inexpensive method widely employed as a readout method for these antibody-based diagnostics. Fluorescent antibodies are incubated with a biological sample, then cells are both counted and analyzed for the presence of fluorescent signal. Cytometry has been widely used for the diagnosis of different diseases, such as detection of biomarkers for various forms of leukemia.[12] The lateral flow assay, or rapid antigen test, is a burgeoning section of the immunoassay field.[13] This technique has been employed to detect cancer markers in blood, urine, and stool, such as elevated Prostate Specific Antigen (PSA) in prostate cancer.[14] A staggering 400 million lateral flow assays are used each year to diagnose malaria in Africa.[15] Additionally, lateral flow is the

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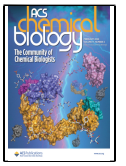





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basis for eight FDA approved SARS-CoV-2 rapid antigen tests which have become key tools in enabling large-scale COVID-19 testing, including the successful campaign to test the entire population of Slovakia within a month.[16,17] The advantages of these assays are their speed (usually 15 min or less), relatively low cost, signal amplification due to secondary antibody binding which helps to increase assay sensitivity, and lack of requirement of highly specialized equipment and personnel to perform and analyze the assay. However, antibody-based assays are not without their drawbacks. They rely on the presence of a specific antigen in patient samples which may not exist in some populations of pathogens, and they often struggle with the detection of infections with low pathogen loads due to overall low sensitivity.[13] For example, the rapid antigen tests for SARS-CoV-2 have a sensitivity of approximately 95% in high viral-load individuals, but the sensitivity drops to only 50−60% in asymptomatic or presymptomatic populations.[18,19]

PCR is often considered the gold standard of diagnostics for several diseases due to the remarkably high sensitivity and specificity of the assay. This method has proven to be highly effective in detection of a variety of clinical indications, including cancers,[20] bacterial infections,[21] and more recently, detection of SARS-CoV-2.[22] A major advantage of PCR is the ability to amplify a signal by a factor of over 1 billon within an hour. However, PCR diagnostics require expensive instrumentation and specialized lab personnel and are often not amenable for use as point of care diagnostics.[23]

Imaging has been a keystone in diagnostics for the last 100 years, especially in the field of cancer.[24] It was observed in the 1920s that tumors tend to uptake more nutrients than healthy tissues.[25] This finding has been exploited to image tumors using radiotracers that measure increased uptake of carbohydrates, nucleic acids, amino acids, and iron via positron emission tomography (PET).[26,27] Another approach has been to conjugate long-lived radionuclides to antibodies that detect upregulated expression of cell-surface proteins, such as EGFR in colorectal cancer, HER2 in breast cancer, and PSMA in prostate cancer.[28] Recent advances in chemical biology have enabled systematic analysis of the downstream effects of a given oncogenetic mutation on cell-surface protein expression,[29] informing antibody-based PET strategies that target novel upregulated cell-surface antigens for both diagnostic and therapeutic purposes.[30,31] Additionally, the advent of MRI and CT imaging modalities in the 1970s and 1980s revolutionized noninvasive imaging of abnormalities in tissue structure.[24] However, such imaging methods do not give detailed molecular information about the microenvironment being imaged. Furthermore, as with PCR-based diagnostics, imaging techniques require expensive, specialized instrumentation and reagents and trained personnel and are low-throughput and time-limited by the need for in-person appointments. Nevertheless, it is an essential tool for noninvasive diagnostics of diseases of difficult-to-access tissues.

Given the challenges and limitations associated with the gold-standard diagnostics described above, activity-based diagnostics have become an attractive alternative. Advances in chemical biology, dye development, substrate development, imaging technologies, and bioengineering have guided the growth of activity-based diagnostics over the past decade. Although there have been several high-quality reviews on this topic, the field is rapidly making significant advancements that have yet to be reviewed in depth. Previous reviews extensively cover caged fluorescent molecules that respond to both upregulated enzymatic activity and inorganic compounds in disease microenvironments, novel "trigger" groups that have been described over the past decade, and the advancement of the field from 2010 to 2019.[2,32,33] Here, we highlight recent methodologies employed in activity-based diagnostics and divide the examples of new chemical entities based upon the modalities used for signal readout. In addition, we cover improvements in past approaches as well as advances in readouts that provide enhanced signal specificity and intensity in complex biological systems.

## QUENCHED FLUORESCENCE

Activity-based diagnostics generally make use of reporter probes that provide a specific signal in the disease tissue of interest. In many cases, probes are designed to contain a fluorophore that is caged such that the signal is only produced upon the action of a disease-associated enzyme. This includes both substrates that are processed by an enzyme as well as quenched activity-based probes (qABPs) that form permanent covalent bonds to target enzymes in the process of signal activation (Figure 1A).[34] Quenched fluorescent probes have

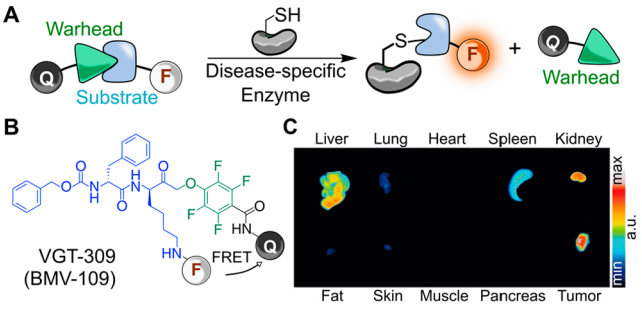


**Figure 1.** (A) The general mechanism of quenched activity-based probes. Q = Quencher. F = Fluorophore. While intact, fluorescence is quenched via FRET. Enzymatic activity results in a covalent fluorescently labeled enzyme and a liberated quencher-warhead sequence. (B) The chemical structure of VGT-309. The fluorophore (F) is ICG, and the quencher (Q) is QC1. (C) *Ex vivo* imaging of tissue 24 h postinjection of 20 nmol VGT-309. Image reprinted with permission from ref 39. Copyright 2020 Springer Nature.

classically relied on the activity of hydrolases including proteases, lipases, and glycosidases to generate a signal, as these classes of enzymes catalyze the cleavage of a chemical bond.[32] However, other types of enzyme activities can be exploited for diagnostic probe development, such as a probe that detects the upregulated oxidation activity of heme oxygenase (HO-1) in cardiovascular and neurodegenerative disease.[35] Here, we will focus both on novel applications and on conjugation strategies used in quenched fluorescent activity-based diagnostics.

In the past decade, qABPs and quenched fluorophores have shown clinical promise in cancer diagnostics by targeting upregulated enzymatic activity in the tumor microenvironment.[36] Specifically, fluorescent probes that respond to upregulated enzyme activity in the tumor microenvironment have great potential for applications in surgical guidance. These quenched probes can facilitate detection of the physical border between tumor tissue and proximal healthy tissue during resection. Traditional surgical methods are informed by classical imaging and surgical expertise. However, relying only on these techniques can often lead to removal of excess


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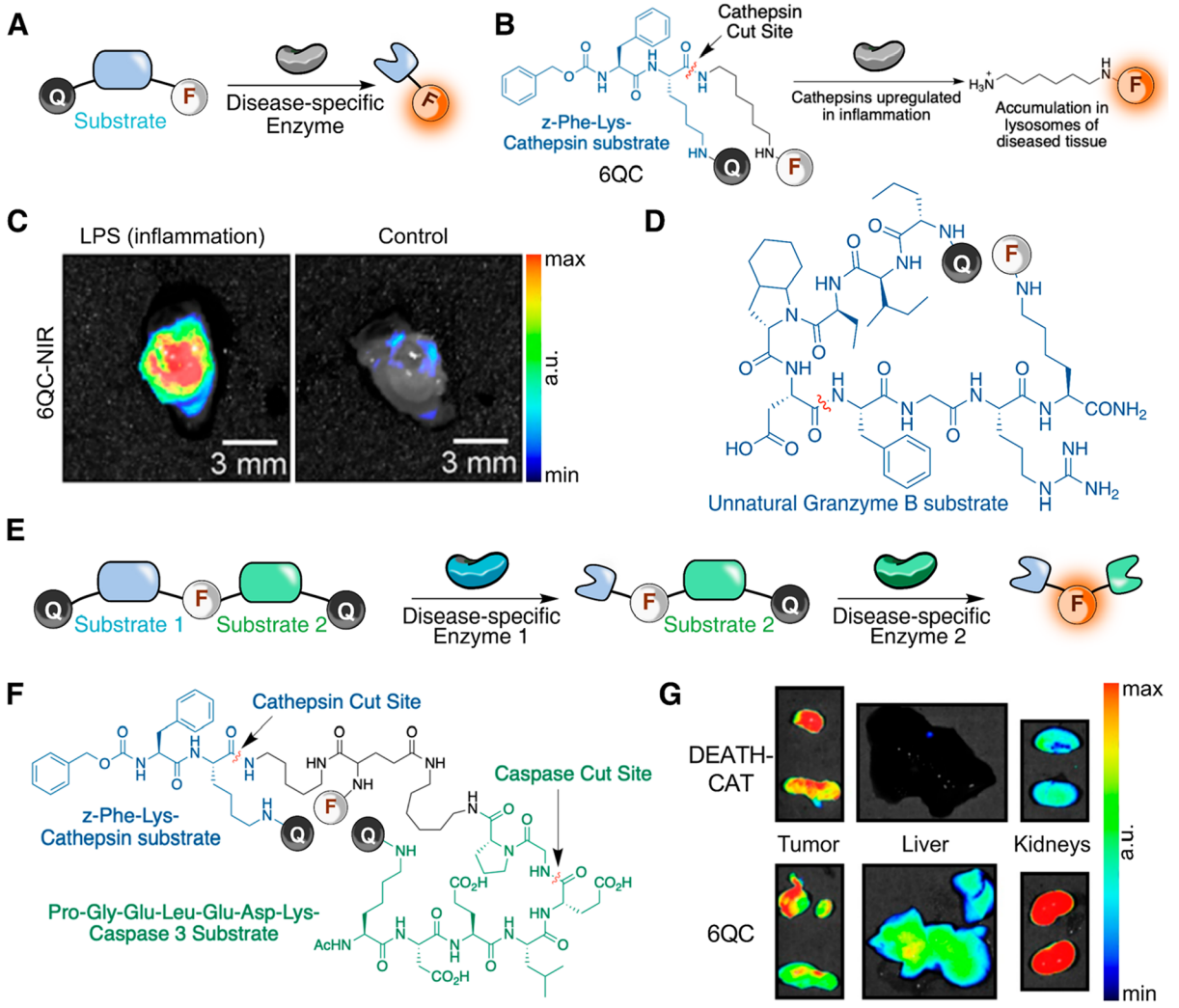


**Figure 2.** (A) General scheme for quenched fluorescent probes. F = fluorophore. Q = quencher. (B) The structure of 6QC. The substrate is processed by cathepsins which are upregulated in disease-related inflammation. (C) 6QC is specifically activated upon LPS-induced inflammation in a murine ear infection model. Image reprinted from ref 43. Copyright 2020 American Chemical Society. (D) Structure of quenched fluorescent probe activated by granzyme. Incorporation of unnatural amino acids greatly increases the specificity of the probe. The red line indicates the site of hydrolysis. (E) General scheme of an AND-GATE approach for quenched fluorescent substrate probes. The fluorophore remains quenched until both substrates have been processed. (F) The structure of DEATH-CAT. (G) Robust activation of DEATH-CAT (Cathepsin and Caspase AND-GATE) in a tumor and reduced fluorescence in background tissues. Image reprinted with permission from ref 46. Copyright 2020 Springer Nature.

healthy tissue or residual tumor tissue being left behind in the surgical cavity. Approximately 20−40% of patients who undergo traditional surgical resection will require a secondary procedure to excise residual tumor tissue that was missed in the first operation.[37] By leveraging enzymatic markers of disease, qABPs can be utilized as a highly sensitive method for detecting cancerous cells in the background of healthy proximal tissues. The qABP VGT-309 (originally published as a Cy5 version BMV-109) is a covalent inhibitor of lysosomal cysteine cathepsins containing an ICG fluorophore and QC-1 quencher pair, separated by a pan-cathepsin peptide substrate and electrophilic warhead (Figure 1B).[38] While the probe remains intact, the emission energy of the ICG fluorophore is donated to the quencher via Förster resonance energy transfer (FRET), upon which the quencher releases energy through nonfluorescent vibrational relaxation. Covalent inhibition of the active site cysteine labels target cathepsins with the peptide-fluorophore conjugate while the quencher sequence is cleared (Figure 1A). Because cathepsins are upregulated in tumor-associated macrophages within the tumor microenvironment, the probe is specifically activated in tumorigenic tissues.[39] VGT-309 efficiently labels tumor tissue in a variety of xenograft preclinical models (Figure 1C) and has recently begun a phase 2 clinical trial as an imaging agent for lung cancer in Australia (ACTRN12621000301864p). As probe activation simultaneously inhibits the enzyme of interest, qABPs can only produce a 1:1 signal to enzyme protein concentration, potentially limiting the contrast that can be obtained in cases where target protein expression is low.

Similar to qABPs, quenched fluorophores leverage the elevated enzymatic activity in tumor tissues; however, unlike qABPs, they offer the potential for signal amplification as multiple substrates can be continuously processed by a single active enzyme (Figure 2A).[36] The quenched fluorophore probe, LUM015, has been evaluated in several clinical trials as a fluorescent tumor contrast agent in patients with a variety of solid tumors (NCT03321929, NCT03717142, NCT03441464, NCT03834272). LUM015 contains a cathe-


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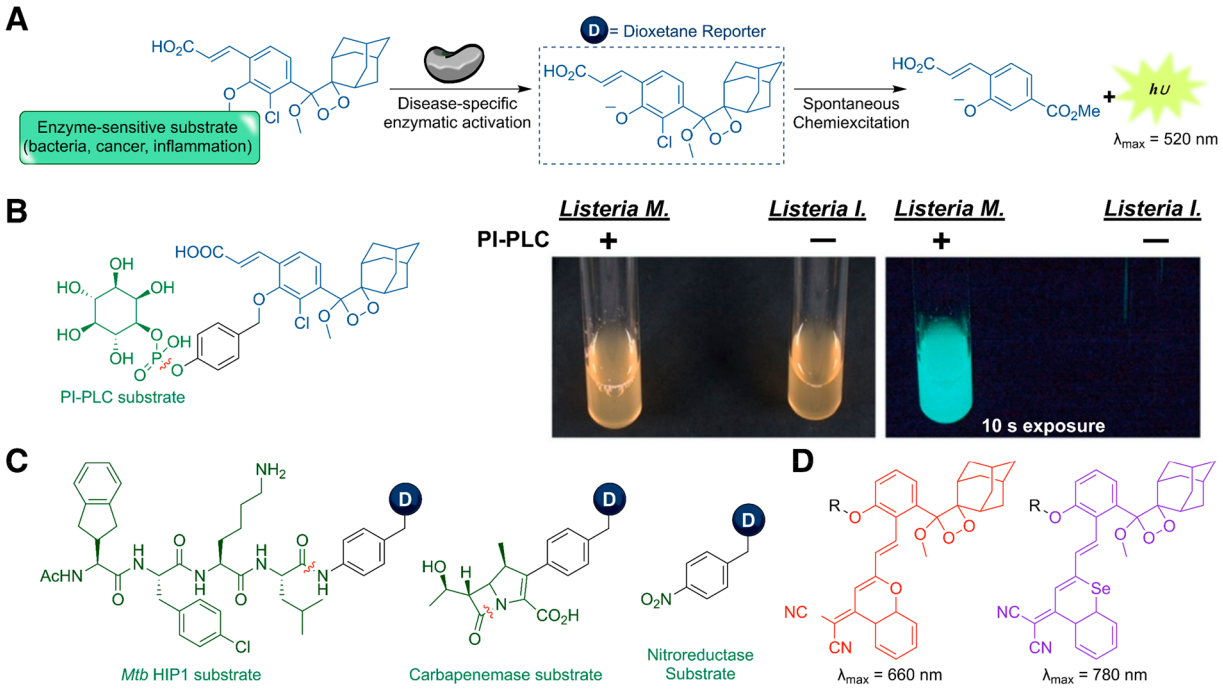


**Figure 3.** (A) General scheme for activation of caged 1,2-phenoxydioxetane chemiluminescent reporters. Upon enzymatic processing, the phenoxy group is released to give an activated dioxetane reporter. Spontaneous chemiexcitation and degradation of the scaffold produces a photon of light. (B) A probe specific to phosphatidylinositol-specific phospholipase C (PI−PLC), present in the pathogenic *Listeria monocytogenes* but not in the nonpathogenic *Listeria innocua*. A 10 s exposure after incubation with 66 $\mu$M substrate demonstrates a high signal and negligible background signal. Image reprinted with permission from ref 63. Copyright John Wiley and Sons 2019. (C) Recent chemiluminescent substrates showcase the versatility of this approach by leveraging the activity of proteases (HIP1), $\beta$-lactamases, and nitroreductase. (D) Novel 1,2-dioxetane scaffolds that shift the emission spectrum toward near IR wavelengths for improved *in vivo* imaging.

psin protease substrate that separates a Cy5 fluorophore and QSY21 quencher pair, along with a 20 kDa polyethylene glycol (PEG) tail to improve pharmacological properties.[40] In a phase 1 clinical trial, the probe was systemically administered to 40 breast cancer patients 1−6 h prior to surgery, and after a standard lumpectomy procedure, surgical cavity tissue was imaged for a fluorescent signal using a hand-held imaging system. The sensitivity of LUM015 for tumor detection in the surgical cavity was 84%.[41] Further, in the eight patients with tumor cells present at the edge of resected tissue who were confirmed to have positive tumor margins, sensitivity for detecting residual tumor cells inside the cavity was 100%. Two of the eight patients with positive margins were spared secondary surgeries because additional tissue was excised at cavity sites with a high LUM015 signal. In two other patients, the lack of fluorescent signal in the surgical cavity correctly identified the absence of tumor cells remaining after the standard surgery. In the remaining four cases, surgeons elected not to resect remaining surgical cavity tissues with a high fluorescent signal, and all four patients eventually required secondary surgical procedures. The high sensitivity of this probe demonstrates the clinical utility of these enzymatic activity-based diagnostic tools; however the specificity of LUM015 was only 73% and falsely led surgeons to remove excess benign tissue in the surgical site.

As an alternative to LUM015, a cathepsin-activated fluorescent peptide, 6QC (Figure 2B), with reduced overall size compared to LUM015, was shown to be an effective preclinical agent for imaging of a range of disease states associated with elevated cathepsin activity, such as inflammation in middle-ear infections (Figure 2C).[42,43] Additionally, the 6QC probe is compatible with the clinically approved da Vinci optical guided surgical system.[44] Though the preclinical results are promising, high levels of active cathepsin activity in healthy organs such as the liver and kidneys result in a high background signal, thus potentially limiting the contrast that can be obtained with the 6QC probe in some types of cancer. One approach to increase the specificity of peptidic probes is to incorporate unnatural amino acids in the substrate sequence. This approach has afforded a wide array of quenched fluorescent probes with exquisite selectivity, such as the recent imaging agent for granzyme B, which includes both norvaline and octahydroindole (Figure 2D).[45]

An additional approach to reduce the background signal in healthy tissues is to use an AND-GATE logic in probe development.[46] This approach involves caging a single fluorophore with two quenchers that are linked by distinct substrate sequences. The AND-GATE logic requires both sequences to be cleaved to produce a detectable fluorescent signal. By selecting substrates for enzymes that are both associated with elevated activity in the tumor microenvironment while never elevated together in healthy tissues, it is possible to generate an exceptionally high level of tumor contrast in virtually any tissue background (Figure 2E). In the initial proof of concept studies, the original cathepsin substrate was coupled with a substrate for an executioner caspase that is only active in dying cells.[47] Because tumors typically have high levels of apoptotic cells which are eventually cleared by circulating macrophages that have high cathepsin activity, these two proteases are ideal targets for the AND-GATE approach.[48] Application of a dual Caspase 3/Cathepsin AND-GATE probe, termed DEATH-CAT, to multiple mouse models of cancers


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greatly decreased the background signal observed in off-target tissues while maintaining a significant signal in tumor tissues (Figure 2F and G). These findings highlight the value of the dual reporter approach for reducing background signals and increasing overall contrast, enabling detection of small tumors in diverse tissue locations.

## CAGED CHEMILUMINESCENCE

In contrast to fluorescence, luminescence is an attractive imaging modality for activity-based diagnostics due to the lack of a background signal in complex biological systems.[49] Significant advancements in exploiting the natural luciferin/luciferase system found in fireflies have provided a variety of caged luciferin analogs that detect specific enzymatic activity both *in vitro* and *in vivo*.[50] Caged luciferin probes have successfully detected elevated enzymatic activity in both solid and blood-borne cancers as well as in bacterial infections, inflammation, and more.[51] Directed evolution of the natural luciferase enzyme coupled with chemical modifications of the luciferin scaffold have produced enzymes capable of producing high wavelength light with improved tissue penetrance.[52] Furthermore, by creating several orthogonal mutant luciferin/luciferase pairs with distinct emission wavelengths, it is possible to perform imaging studies and obtain spatial and temporal readouts of multiple enzymatic activities *in vivo*.[53] However, these studies require the engineered expression of luciferase for signal generation, which limits clinical utility for *in vivo* imaging. Injectable luciferase plugs address this issue in preclinical murine models. However this technology currently has limited clinical utility as the implantable luciferase plug can only measure the amount of uncaged luciferin in the bloodstream and does not provide spatial information on where enzymatic uncaging occurred in the body.[54] Chemiluminescence is an attractive alternative to the luciferin/luciferase technology as the luminescent signal is generated spontaneously through a chemical reaction that does not require a secondary enzymatic reaction to produce light after uncaging.[55] Thus, it becomes possible to cage the chemiluminescent substrate such that upon processing by an enzyme of interest (e.g., cancer associated protease) the molecule is turned over to produce a photon of light. This is desirable for imaging as these chemiluminescent signals can be detected rapidly and portably with inexpensive and easy to use devices.[54,56] Caged luminescent probes have been utilized for enzymatic detection for several decades. However the uncaged luminescent reporters were generally not optimized for most biological applications.[57] Recent work on optimizing Shaap's 1,2-dioxetane to improve aqueous solubility and quantum efficiency has produced a versatile scaffold for caged chemiluminescent probes for use in cellular and *in vivo* applications (Figure 3A).[58] Caging of the luminescent signal is classically performed by conjugating the substrate directly to the phenoxy-group, such as phosphorylating the phenol to afford a probe that detects alkaline phosphatase activity.[59] Recent work on coupling the phenol to benzylic linkers has greatly expanded the scope of substrates that can be used with this platform (Figure 3B).

Applications of these caged dioxetanes have proven to be useful for a variety of preclinical models. Similar to quenched fluorophores, the dioxetanes remain inert until processing by enzymes that are upregulated in the pathology of interest. For nontransmissible human diseases, chemiluminescent reporters have been reported that detect elevated enzymatic activity of granzyme B in tumor associated NK cells,[60] cathepsin activity in tumor microenvironments,[61] and PSA in prostate cancer.[62]

Importantly, this system is remarkably stable to complex biological systems that do not contain the enzymatic activity of interest, as demonstrated by a dioxetane moiety attached to a substrate specific to phosphoinositide phospholipase C (PI−PLC).[63] When the resulting probe was incubated with multiple species of *Listeria*, the luminescent signal was only observed in species that express PI−PLC, such as the pathogenic *L. monocytogenes*, while no background signal is observed in *L. innocua* which does not express PI−PLC (Figure 3B). Therefore, caged luminescent scaffolds can remain intact in complex biological systems and, with a specific substrate, only produce a signal in the presence of the target.

This platform can accommodate a variety of substrates that target several classes of enzymes (Figure 3C).[64−66] A probe with a peptide substrate specific for *Mycobacterium tuberculosis* enzyme HIP1 provides a promising diagnostic signal in patient sputum.[67] The probe also allows rapid assessment of drug susceptibility testing without the need to do long-term culture studies, enabling rapid decision making about types of antibiotics to use for treatment. Similarly, a probe that contains a general carbapenem substrate can inform on the presence of carbapenemase activity in bacterial infections, providing physicians crucial information on drug-resistance profiles. The platform is not limited to hydrolytic enzymes. However, a recent probe was described that detects elevated nitroreductase activity *in vivo* in hypoxic microenvironments associated with solid tumors.

Recent advances in the dioxetane technology have improved the *in vivo* imaging potential of the platform. Self-immolating dioxetane polymers can be used to amplify the signal when enzymatic activity levels in disease tissue are low.[68] To achieve this amplification, a single substrate cages a polymer of 20 or more dioxetane reporter molecules. After enzymatic processing of the substrate, the self-immolative polymer rapidly degrades and produces a signal that is proportional to the number of dioxetane molecules released. Modification of the dioxetane scaffold has afforded novel reporter molecules that produce a higher wavelength IR or NIR emission to improve tissue penetrance (Figure 3D), although examples of the application of these scaffolds for *in vivo* diagnostics have not yet been reported.[69,70]

## COLORIMETRIC

Colorimetric assays are highly desirable for point of care diagnostics as they do not require complex instrumentation to analyze a result.[71] The enzyme-linked immunosorbent assay (ELISA) is a prominent example of a colorimetric assay readout. Typically, the presence of a particular antigen in a biological sample is determined by an antibody linked to a secondary enzyme, such as horseradish peroxidase (HRP), that will process an assay reagent, such as 3,3′,5,5′-tetramethylbenzidine (TMB), resulting in a color change.[72] However, this type of assay requires an abundance of the antigen of interest.[71]

Several diagnostic tools have been developed for colorimetric detection of enzymatic activity without the need for antibodies. Many of these assays rely on the enzymatic reduction of tetrazolium dyes, such as the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to the visibly purple formazan.[73] This method was recently employed to detect succinate dehydrogenase activity in sperm, as low enzymatic activity is a biomarker for low total motile sperm


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concentration (TMSC).[74] Additionally, MTT and its analogs have been shown to be processed in a variety of bacterial infections, including sepsis,[75] UTIs,[76] and more recently *Neisseria gonorrheae*.[77] However, the clinical application of these dyes is limited due to the lack of specificity to any one particular indication, potentially confounding results in complex biological samples.[78]

A limitation of colorimetric diagnostic assays is the amount of signal required to be readily viewed by the eye.[71] For example, the nitrocefin assay (Figure 4A) becomes red upon

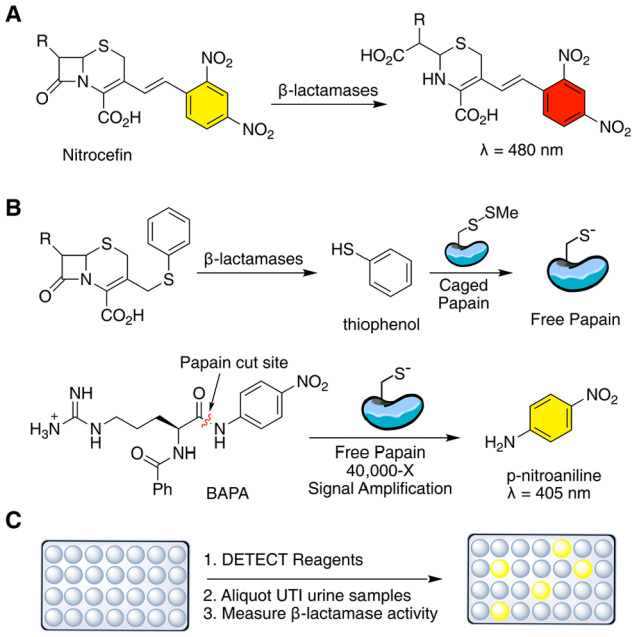


**Figure 4.** (A) Processing of nitrocefin via $\beta$-lactamases. (B) Scheme of DETECT dual-enzyme signal amplification. Thiophenol is released upon $\beta$-lactamase activation. Thiophenol then selectively activates a disulfide-caged papain, which processes over 40,000 additional copies of BAPA to produce a yellow readout. (C) General workflow of the DETECT assay in a plate-based format.

processing via beta-lactamases. However this assay requires a high concentration of enzymes to give a sufficient signal to be observed by eye.[79] As such, nitrocefin assays are performed on samples that are first cultured to increase the bacterial concentration, thus lengthening the time to diagnosis by several days.[7] This issue can be addressed by utilizing a dual-enzyme based colorimetric assay for beta-lactamases termed DETECT.[7] The assay is composed of a thiophenol-bearing cephalosporin analog, a disulfide-caged papain, and N-benzoyl-L-arginine-para-nitroaniline (BAPA). Upon processing of the cephalosporin by beta-lactamases, thiophenol is released into solution, which selectively uncages papain. As the newly freed papain processes BAPA to para-nitroaniline, the solution turns yellow (Figure 4B and C). By utilizing a secondary enzyme, the signal generated is amplified by 40,000-fold, enabling the DETECT assay to measure the presence of beta-lactamases in uncultured urine samples from UTI patients in just 30 min with a sensitivity and specificity of >90%.[80]

The DETECT assay shows promise for use in combination with other bacterial diagnostic assays. The current gold-standard to determine antibiotic resistance of a clinical bacterial isolate involves first culturing the bacteria before performing minimum inhibitory concentration (MIC) assays, which often requires 48−72 h to provide antibiotic susceptibility testing (AST) information.[81] Instead, utilizing the DETECT system as a readout for antibiotic susceptibility testing (AST-DETECT) drastically reduces the time to 3 h due to its remarkable sensitivity in low-density bacterial cultures.[82] AST-DETECT is performed by monitoring the change in DETECT score for a given bacterial sample pre- and post-treatment with an antibiotic. The DETECT score decreases significantly in 2 h if the bacteria are susceptible to the antibiotic treatment, while resistant bacteria show no significant change in score. AST-DETECT can accurately identify antibiotic resistances to fluoroquinolones, cotrimoxazole, and nitrofurantoin with high sensitivity and specificity in both laboratory and clinical isolates.

## MULTIMODAL NANOSENSORS

In addition to small molecule diagnostics, injectable activity-based nanosensors (ABNs) are a new class of diagnostics that leverage dysregulated enzymatic activity in diseased tissue for diagnostic purposes. The recently reported Protease-Responsive Imaging Sensors for Malignancy (PRISM) is an example of such technology and utilizes injectable iron-oxide nanoparticles decorated with various peptide sequences that leverage dysregulated metabolic activity in the tumor microenvironment (Figure 5A and B).[83,84] In particular, PRISM probes have exploited elevated activity of matrix metallopeptidase 9 (MMP9) due to their role in the tumor "angiogenic switch" found in diverse types of cancer.[83] By conjugating peptide sequences to these nanoparticles which couple MMP9-specific substrates with peptide sequences that direct hydrolyzed products toward renal clearance, a variety of xenograft and spontaneous tumor models have been sensitively detected by monitoring urine for cleaved MMP9 peptides via mass spectrometry or ELISA (Figure 5C).[85,86] Although this method enables noninvasive diagnosis of cancer, it provides no spatial location of where enzymatic processing of the MMP9 substrate occurred *in vivo*. To address this limitation, fluorescein amidite (FAM) was conjugated to the iron oxide nanoparticles through an MMP9-specific substrate, resulting in accumulation of the FAM signal in the margins of the tumor in several xenograft models (Figure 5D).[87] This colocalization is further improved *in vivo* through additional conjugation of tumor-directing peptides onto the nanoparticles, such as an acidosis-mediated tumor targeting sequence.[88] Directing the *in vivo* localization of these nanoparticles improves the diagnostic ability of the platform by concentrating the ABNs to tumor tissue and thereby decreasing the chance of off-target processing in healthy tissues that also express MMP9. Conjugation of $^{64}$Cu, a PET compatible radionuclide, to the terminus of the tumor-targeting peptide highlights the significantly increased tumor localization of these nanoparticles (Figure 5E). Further development of this technology will likely yield iron-oxide nanoparticles that can perform multiplexing and AND-GATE logic strategies. While the PRISM platform is currently being evaluated in multiple preclinical cancer models, a similar nanosensor platform has been advanced into phase 2 clinical trials for the detection of Non-Alcoholic Steatohepatitis (NASH) by Glympse Bio.

## FUTURE OUTLOOK

Through recent advances in chemical biology, dye development, bioengineering, and imaging modalities, activity-based


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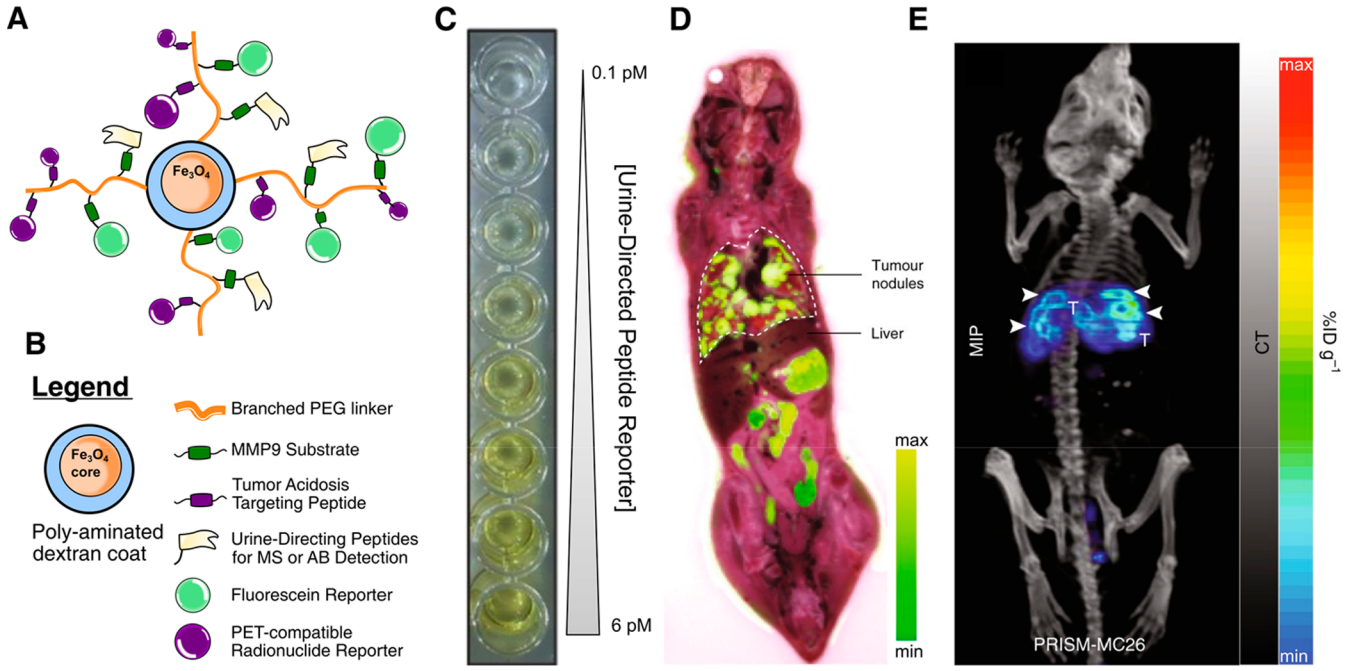


**Figure 5.** (A) Representative activity-based nanosensor containing multiple reporter systems. (B) Potential components of iron-oxide based nanoparticles for multimodal readouts. (C) An ELISA-based readout of urine-directed peptide reporters with a limit-of-detection of approximately 1 pM. Image reprinted with permission from ref 86. Copyright 2014 The National Academy of Sciences. (D) Fluorescent FAM signal accumulates in tumor nodules with elevated MMP9 activity. (E) Representative maximum intensity projections for PET imaging 2 days postinjection of PRISM nanosensors conjugated with $^{64}$Cu radionuclide. White arrows and T mark colorectal cancer (CRC) liver tumors. Images D and E reprinted with permission from ref 84. Copyright Springer Nature 2021.

diagnostics have become increasingly valuable as point of care tools. The advancement of substrate profiling has provided a multitude of highly specific probes for elevated enzymatic activity related to both infectious and nontransmissible diseases including cancer, bacterial infections, inflammation, and more. In cases where target enzymatic activity is also observed at high basal levels in healthy tissues, leveraging AND-GATE logic strategies that require the presence of an orthogonal disease-specific enzyme will help to produce specific probes with greatly improved signal contrast. Continual improvements in specificity of substrates may soon enable small molecule probes to determine the genus and species of a particular infectious pathogen as well as its drug resistance and susceptibility profiles. These types of tools would be paramount to the advancement of precision medicine for infectious disease. Additional work is ongoing to utilize highly selective probes to inform the development of similarly selective inhibitors, thereby opening the door to therapeutic-diagnostic (theranostic) agents.

Along with advancements in probe design, novel advances in imaging modalities are enabling an increased array of potential readouts for activity-based diagnostics. An increasing large number of conjugatable dyes are becoming commercially available for use in fluorometric-based probes with clinically desirable properties such as high quantum yields, NIR and IR emission wavelengths for better tissue penetrance, and better alignment with the excitation and emission properties of FDA-approved optical-guided surgical cameras. Addressing current limitations of *in vivo* imaging has also fueled the emergence of chemiluminescence as an attractive modality for activity-based diagnostics. The lack of excitation source (such as laser in fluorescence or luciferase in the luciferin platform) provides a multitude of advantages in regard to sensitivity, especially due to the negligible background signal observed in human tissues. Furthermore, it greatly simplifies the diagnostic test, thus making it more accessible in a resource-limited setting.

Although colorimetric readouts for diagnostic assays are desirable due to the lack of instrumentation or expertise necessary to interpret the results, generation of sufficient signal for visible color change is challenging in infections with low pathogen levels, or when the expression of the target enzyme is low. Traditionally signal-to-noise issues in colorimetric assays have been resolved by culturing a clinical sample to increase enzyme concentration. However the time commitment for this step, along with the large number of pathogens without an appropriate culture technique, leave an unmet need in the field. Novel signal amplification strategies, such as the dual-enzyme DETECT assay, can improve the limit of detection by more than 4 orders of magnitude, enabling the assay to be performed directly on the clinical sample, thus reducing time to diagnosis to less than an hour.

By leveraging the advancements in fields such as chemical biology and bioengineering, novel activity-based diagnostics are proving to be promising contributors to the clinical diagnostic field. For example, the combination of ABPs with novel mass cytometry technologies, such as CyTOF (Cytometry by Time-Of-Flight), has recently enabled the multiplexed analysis of four enzyme activities at single-cell resolution within the same sample.[89]

These technologies are addressing several challenges that face traditional diagnostic modalities. The increased sensitivity provided by quenched fluorescent probes aims to provide surgeons with guidance to allow them to perform more accurate tumor resections. The ease and portability of chemiluminescent readouts coupled with the lack of background signal in biological samples enable these probes to fill


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an unmet need in rapid, portable diagnostics. Meanwhile, using strategies to amplify the signal by harnessing enzymatic turnover will increase sensitivity in traditional modalities that already possess desirable attributes, such as the ease of interpreting results for colorimetric readouts. Finally, the ability to couple multiple readouts to activity-based nanosensors will provide physicians with diverse information through noninvasive measures. The preclinical successes and advancement of platforms to the clinic, such as fluorescent-guided surgery and activity-based nanosensors, are encouraging for the future of activity-based diagnostics for a variety of disease states. However, no clear path yet exists for these platforms to advance through the clinic, and therefore additional efforts will be required to translate new activity-based diagnostic tools from bench to bedside.

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## KEYWORDS

**Activity-based diagnostics**: a class of chemical probes that produce a detectable signal in the presence of enzymatic activity in disease state tissue.

**Signal amplification**: A strategy employed in diagnostics to boost the detectable signal, such that many detectable molecules are generated from a single biomolecule of interest. For example, an enzyme can process many thousands of molecules of a fluorescent substrate.

**Point-of-care diagnostics**: Diagnostics that can be performed rapidly at the location in which the biological sample is obtained. This contrasts with classical methods in which biological samples are collected, then sent to a medical testing laboratory in which the diagnostic assays are performed.

**FRET**: Förster resonance energy transfer; describes the energy transfer between two chromophores where the emission spectrum of the FRET donor overlaps with the excitation or absorption spectrum of the FRET acceptor

**Sensitivity**: The ability of a particular diagnostic to correctly identify true-positive samples

**Specificity**: The ability of a particular diagnostic to correctly discern true-negative samples

**qABP**: Quenched activity-based probes. A class of activity-based diagnostics containing a fluorophore and quencher pair, separated by an enzyme substrate and electrophilic warhead. Once the enzyme of interest acts upon the qABP, the enzyme is covalently labeled with the fluorophore while the quencher is released.

**Chemiluminescence**: A phenomenon in which a photon of light is produced as a result of a chemical reaction.

**Multimodal diagnostics**: A class of diagnostics in which multiple readout modalities are employed; this can include turn-on fluorescence, chemiluminescence, mass-spectrometry tags, and more.

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