

# Evaluation of Enzymatic Activities in Living Systems with Small-molecular Fluorescent Substrate Probes

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In this review, we aim to present an overview of how small-molecular fluorescent substrate probes for studying enzymatic functions are developed and how they are used in biological applications, under the following four headings: (1) History of Visual Detection of Enzymatic Activities, (2) Strategies to Design Fluorescent Substrate Probes to Measure Enzymatic Activities, (3) Development of Fluorescent Substrate Probes Suitable for Biological Studies, and (4) Biological Applications of Fluorescent Substrate Probes for Studying Enzymes.

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## 1 Introduction

Recent progress in genomics has shown that the human genome

comprises more than 20000 genes,<sup>1</sup> of which approximately half are still not fully characterized. Further, the proteome is far larger than the genome, due to alternative splicing and post-translational modifications,<sup>2</sup> and the biological functions of



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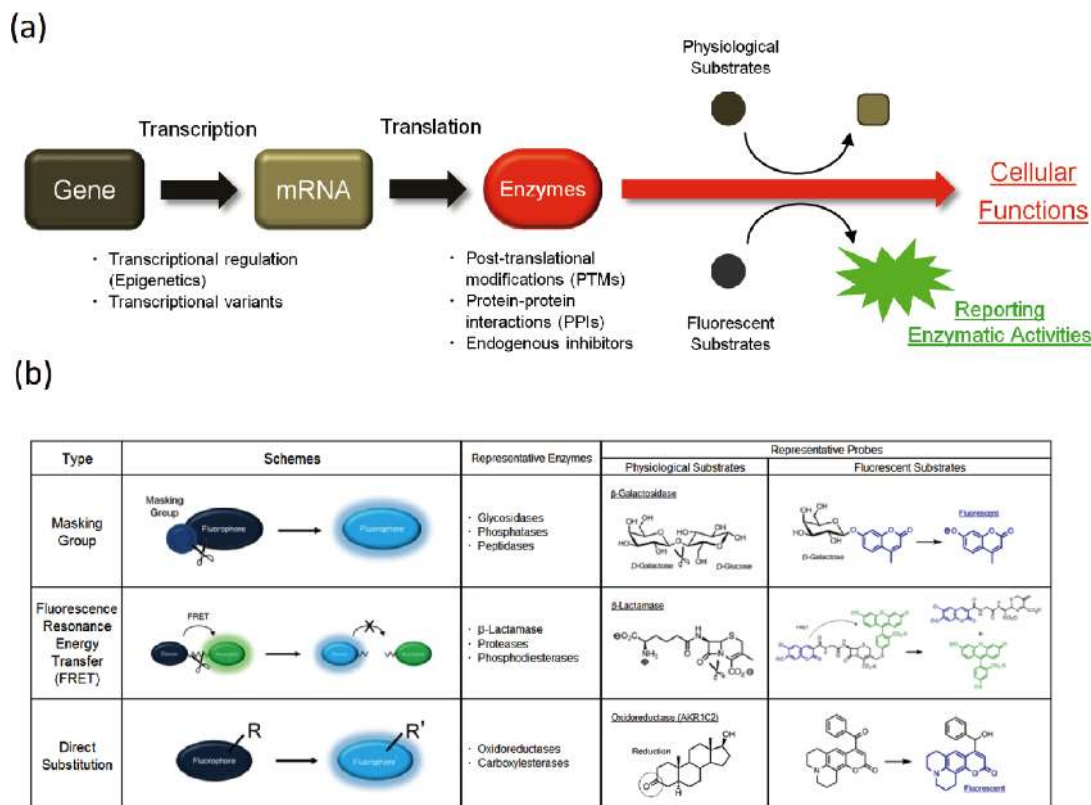


Fig. 1 (a) Schematic illustration of the use of enzymatic activity-reporting substrates for functional analysis of proteins. In living cells, functional activities of proteins can be regulated at the DNA (gene) or mRNA or protein level. A fluorescent reporter substrate can directly visualize the step in which an enzyme exerts its physiological function by metabolizing its substrates. (b) Schematic illustration of major design strategies and their applications to fluorescent substrates. See the text for details and references.

many proteins, as well as the changes of their functional activity under various pathophysiological conditions, remain largely unknown. Thus, it is clear that the number of potential drug targets is enormous.<sup>2,3</sup> Currently, more than 90% of drug targets are proteins, and among them, enzymes form one of the most important groups.<sup>2</sup> Aberrant activity of enzymes is often associated with the onset or progression of diseases, so a detailed understanding of enzyme functions, together with tools to measure enzymatic activities, is of great importance for diagnosis and treatment of diseases. However, one of the difficulties in establishing the functions of enzymes is that they work in a dynamic manner in living systems,<sup>4</sup> and their activity is usually modulated by multiple factors, including post-translational modifications,<sup>5</sup> protein-protein interactions,<sup>6</sup> and endogenous inhibitors.<sup>7</sup> One of the most useful methods to study enzymatic activities in the biological context is the use of small-molecular substrate probes to directly visualize their catalytic activity<sup>8</sup> (Fig. 1a). For this purpose, substrate molecules that are designed to show a signal, such as a fluorescence change, in response to the reaction catalyzed by the target enzyme, are required. Since fluorescent substrate probes can provide extremely high sensitivity, they are effective tools to study the functions of enzymes *in vitro*, *in cellulo*, and *in vivo*.

In this review, we aim to present an overview of how small-molecular fluorescent substrate probes to study enzymatic activities are developed and how they are used in biological applications, under the following four headings: (1) History of

Visual Detection of Enzymatic Activities, (2) Strategies to Design Fluorescent Substrate Probes to Measure Enzymatic Activities, (3) Development of Fluorescent Substrate Probes Suitable for Biological Studies, and (4) Biological Applications of Fluorescent Substrate Probes for Studying Enzymes. In addition to small-molecular probes, fluorescent protein-based sensors<sup>9</sup> are often used to report enzymatic activities in cell biology. However, here we limit our focus to small-molecular probes since biosensors based on fluorescent proteins have been well reviewed elsewhere.

## 2 History of Visual Detection of Enzymatic Activities

The idea of visualizing enzymatic activity by reporter substrates was established in 1939,<sup>10</sup> when Gomori developed a method to visualize phosphatase activity in tissue sections, based on the idea that phosphate released from glycerophosphate *via* enzyme catalysis could be detected in terms of the formation of an insoluble salt with calcium ions. The idea of detecting enzymatic activity in tissues was followed up by other researchers, aiming to improve the sensitivity and speed of detection, and this work led to the development of various selective colorimetric<sup>11</sup> and fluorometric<sup>12</sup> substrates that measure enzymatic activities in terms of a color change. These assays were used mostly to identify novel enzymatic activities in tissue extracts,<sup>11b,13</sup> and some of the enzymes thus characterized were later established

as potential drug targets. For example, Glenner *et al.* identified Gly-Pro- $\beta$ -naphthylamide-cleaving activity in mammalian tissues in 1966,<sup>14</sup> and purification of the responsible protein led to the identification of dipeptidyl peptidase IV (DPPIV).<sup>15</sup> This enzyme was subsequently identified as a major metabolizing enzyme of incretin,<sup>16</sup> and was established as one of the most promising drug targets for treatment of diabetes.<sup>17</sup>

In recent years, progress in the field of fluorescence imaging has greatly extended the range of applicability of fluorescent substrates. An important advance was made when Tsien *et al.* developed a sensitive  $\beta$ -lactamase substrate probe based on the fluorescence resonance energy transfer (FRET) mechanism.<sup>18</sup> This cell-permeable fluorescent substrate was sufficiently sensitive to quantify enzymatic activity at the single cell level, and this opened up the field of enzymatic activity imaging as a tool for studying cellular functions. Since then, many fluorescent substrate probes have been designed for fluorescence imaging in living cells and living animals,<sup>8a</sup> making it possible to investigate the physiological functions of various enzymes *in situ*.

Thus, the idea of using fluorescent substrate probes to study enzymatic function is not new, but recent progress in instrumentation and improvement of design strategies has led to the introduction of many novel fluorescent substrates that are suitable for biological applications. In the next section, we present a brief overview of the currently available strategies to design fluorescent substrates.

### 3 Strategies to Design Fluorescent Substrate Probes to Measure Enzymatic Activities

In designing fluorescent substrates for enzymes, two key questions have to be considered. (1) How is the substrate analogue metabolized by the enzyme? and (2) How is the metabolizing reaction translated into a change of fluorescence character of the substrate? There are currently several different mechanisms available for fluorescence control, and each has a preferred range of reactions to which it can be applied. In this section, we briefly describe how knowledge of the biochemical reactions of physiological substrates can be applied to design fluorescent substrates (Fig. 1b).

#### 3-1 Masking group

Based on their reaction mechanisms, enzymes have been classified into six families, *i.e.*, oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.<sup>19</sup> Many design strategies have been developed for hydrolases,<sup>8c,8e</sup> since they are highly active, and usually cause dramatic changes of the substrate structure. Hydrolases can be divided into two categories based on the nature of their substrate recognition: exo-type reaction and endo-type reaction (not to be confused with exo-/endo-enzymes, referring to extracellular/intracellular enzymes).<sup>20</sup> In exo-type reactions, hydrolases recognize and cleave the end part of substrates, while in endo-type reactions, they cleave the substrate in the middle of the molecule. In designing fluorescent substrates for enzymes that catalyze exo-type reactions, the key structure of the substrate, which is strictly recognized by the enzyme, is attached to the fluorophore in such a way as to mask the original fluorescence, so that release of the masking group by the enzyme-catalyzed reaction results in recovery of fluorescence. There are several fluorophores that exhibit reduced fluorescence when their structure is modified with masking groups; for example, transformation of phenolate to phenyl ether, or aniline to anilide.<sup>12b,21</sup> This strategy has been used to develop reporters for

various target enzymes, such as glycosidases,<sup>12c,21</sup> phosphatases,<sup>11a</sup> and peptidases.<sup>8c,22</sup> One example is the case of  $\beta$ -galactosidase, an enzyme that is widely used in reporter gene assay.<sup>11c,23</sup>  $\beta$ -Galactosidase cleaves the glycoside bond of lactose to generate galactose and glucose. While the structure of glucose is not critical for substrate recognition, the enzyme strictly recognizes galactose, so galactose can be used as a masking group to develop a fluorescent sensor.<sup>12c,21,23b</sup> For example, fluorescein di- $\beta$ -D-galactopyranoside<sup>21</sup> is a useful fluorescent substrate for  $\beta$ -galactosidase, being cleaved by  $\beta$ -galactosidase to release fluorescein, the intact fluorophore. The advantage of this type of design strategy is that the masking group, galactose here, can be readily applied to develop a range of reporter substrates. Many chemical mechanisms to generate a signal change from an exo-type reaction have been developed, including formation of diazo dyes,<sup>11a</sup> formation of colorimetric aggregates (X-Gal),<sup>11c</sup> color change of 4-nitrophenol derivatives (4-nitrophenyl  $\beta$ -D-galactopyranoside),<sup>24</sup> chemiluminescence reaction,<sup>25</sup> bioluminescence reaction,<sup>26</sup> and contrast enhancement in magnetic resonance imaging (MRI).<sup>27</sup> Also, one can develop substrate probes with additional functions by means of chemical modifications as long as the masking group is properly introduced. For example, increased cellular retention,<sup>28</sup> covalent modification of proteins,<sup>29</sup> and development of probes working over multiple color ranges<sup>30</sup> have been achieved based on chemical modifications of sensor molecules.

#### 3-2 Fluorescence resonance energy transfer (FRET)

Although the masking group strategy described above works well for exo-type enzymes, endo-type enzymes cannot be targeted in this way, since endo-type enzymes usually recognize both sides of the cleavage site, and direct attachment of fluorophores may block enzymatic recognition. For visualizing endo-type reactions, a fluorescence resonance energy transfer (FRET) mechanism is usually employed.<sup>18,29b,31</sup> In FRET-based substrate probes, excitation energy of the donor fluorophore is transferred to an acceptor fluorophore before emission. Since the efficiency of energy transfer is dependent on the distance between the donor and acceptor, molecules in which the donor and acceptor moieties are connected *via* a cleavable linker can report the linker cleavage reaction in terms of a fluorescence spectral change. This design has been employed to develop fluorescent sensors for enzymes catalyzing endo-type reactions, including  $\beta$ -lactamase,<sup>18</sup> proteases,<sup>31a,31d</sup> and phosphodiesterases.<sup>31b</sup> A major advantage of FRET-based sensors is their suitability for ratiometric imaging, where two signals are simultaneously acquired and used to calculate fluorescence ratios; this increases the accuracy of measurement by automatically compensating for changes of dye concentration, reaction volume, and excitation intensity.<sup>18,31c</sup> Ratiometric measurement is especially advantageous for enzymatic activity monitoring in complex biological systems such as live cells.

#### 3-3 Direct conversion of fluorophores

As described above, many design strategies have been developed for visualizing hydrolases. However, other enzyme classes such as oxidoreductases and transferases also have important functions in living systems, and fluorescent sensors are also required to visualize their activities.

Oxidoreductases are enzymes that catalyze oxidation and reduction of specific functional groups on substrates.<sup>32</sup> In order to visualize reactions catalyzed by oxidoreductases, conversion of a single substituent without bond cleavage, such as ketone to alcohol, should be translated into a fluorescence change of the substrate probe. One useful strategy is to introduce the reaction

site directly into the fluorophore, so that the enzymatic reaction causes conversion of the fluorophore itself.<sup>63,64</sup> Since the fluorescence character of some of the fluorophores can be dramatically influenced by single substitution, simple conversion of the substituent group of those fluorophores can be enough to trigger a large fluorescence increase. Sames *et al.* successfully applied the direct conversion strategy to develop a series of substrate probes for oxidoreductases,<sup>32</sup> in which conversion of ketone to alcohol on a modified coumarin scaffold is detected as a dramatic change of fluorescence intensity.

Here, we have briefly overviewed the established strategies that are commonly applied to design fluorescent substrate molecules. However, this is an active area of research, and many new design strategies are under development, including photoinduced electron transfer (PeT)-based probes,<sup>23b,28,33</sup> spirocyclization,<sup>34</sup> self-quenching-based probes,<sup>8h,35</sup> environment-sensitive dyes,<sup>36</sup> control of cellular retention,<sup>37</sup> and aggregation-induced emission (AIE).<sup>38</sup>

#### 4 Development of Fluorescent Substrate Probes Suitable for Biological Studies

The strategies described above can be employed to design fluorescent substrate probes suitable to report various enzymatic reactions *in vitro*. However, in applying those substrate probes for *in cellulo* or *in vivo* study, the substrate probe is required to report the fluorescence signal selectively by the target enzyme in the system containing thousands of different enzymes.<sup>8e,39</sup> Therefore, one of the major challenges in designing fluorescent substrates able to acquire biologically meaningful data in living systems is achieving sufficient specificity to report the function of the target enzyme even in the presence of multiple enzymes of the same enzyme class.<sup>40</sup> Here, we describe strategies to achieve this, with illustrative examples.

##### 4.1 Diversity-based approach

The development of completely selective fluorescent substrates is extremely challenging; the difficulty is comparable to the development of selective pharmacological inhibitors. Therefore, researchers usually apply a library-based approach, in which panels of fluorescent substrates are prepared for screening to select the best ones for the target. Ellman *et al.* developed a systematic approach to find selective fluorescent substrates for peptidases and proteases.<sup>8c,22,41</sup> The basic idea is to prepare a panel of enzymes and a panel of peptide-based substrate probes, which are screened to find the most suitable pair (Fig. 2a). While the enzyme panel does not cover the whole proteome, and the strategy still leaves open the possibility that the selected probe may be cleaved by unpredicted enzymatic activities in living systems, this is probably the most powerful strategy currently available for establishment of specific turnover-based substrate probes for individual enzymes. This strategy has been applied to develop fluorescent substrates for oxidoreductases as well.<sup>32,42</sup>

Another sophisticated strategy for diversity-based development of fluorescent substrate probes was described by Tsien *et al.*, who employed a phage library to identify peptide sequences that are preferentially cleaved in tumor tissues.<sup>43</sup> Evolutional selection was applied to amplify phages bearing suitable peptide sequences, resulting in successful identification of novel substrate peptide sequences for enzymes upregulated in tumor tissues (Fig. 2b). An advantage of this method is that it allows the discovery of both novel biomarkers and selective sensor molecules for them. While this strategy can be applied only to

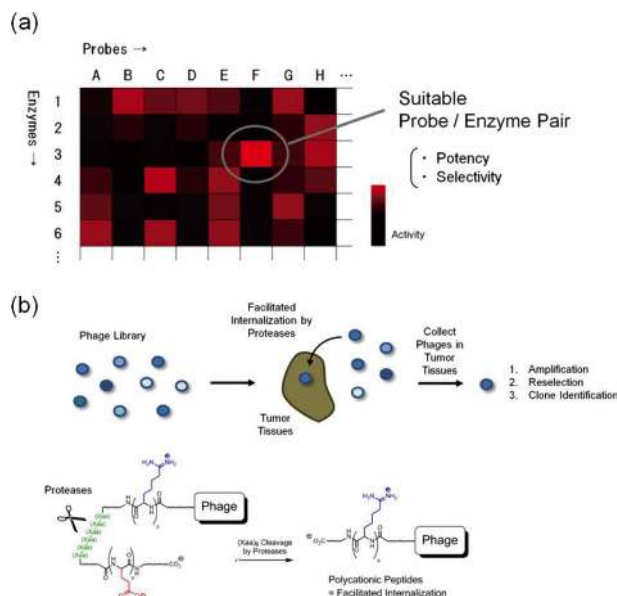


Fig. 2 (a) Schematic illustration of strategies to identify selective turnover-based fluorescent substrates for specific enzymes with the use of panels of candidate probes and panels of enzymes. The signal intensity and the selectivity among enzymes in the panel are taken as criteria to select suitable pairs. (b) Concept of phage selection-based identification of hexameric peptide sequences that are preferentially cleaved in tumor tissues. Phages were designed to express proteins consisting of polycationic peptides and polyanionic peptides connected *via* a variable hexameric peptide linker. Cleavage of the hexameric peptide linker exposes the polycationic peptide and facilitates internalization of the phage. Thus, amplification of phages in the target tissues makes it possible to identify hexameric peptides that are preferentially cleaved in tumor tissues.

proteases, it can be used to develop fluorescent substrates even for uncharacterized targets.

##### 4.2 Fine tuning of substrates based on biochemical knowledge

In some cases, profound biochemical knowledge of the target enzyme can increase the chance of obtaining selective fluorescent substrates without applying the library-based approach. One example is the development of a fluorescent sensor for NPP6.<sup>44</sup> Nucleotide pyrophosphatases/phosphodiesterases (NPPs) are enzymes that cleave phosphodiester bonds of phospholipids.<sup>45</sup> Currently seven members have been characterized for this family, and each of them is reported to have different substrate preferences. Among them, NPP2 and NPP6 recognize phosphatidylcholine,<sup>46</sup> so introduction of a choline group into the substrate probe was expected to generate substrates of these enzymes. These two enzymes differ in how they recognize the substrates. NPP2 is known to catalyze phospholipase D (PLD)-type reaction, while NPP6 has phospholipase C (PLC)-type activity. So, a fluorescent substrate probe was designed to be susceptible to PLC-type reactions, and indeed showed high selectivity for NPP6 over other NPP family members (Fig. 3a). This probe was shown to be useful to detect NPP6 activity in living cells.

Another approach to develop selective fluorescent substrates in an evidence-based manner is the conversion of selective inhibitors to fluorogenic substrates.<sup>47</sup> In this approach, knowledge acquired during inhibitor development, such as structure-activity relationships and selectivity, can be fully

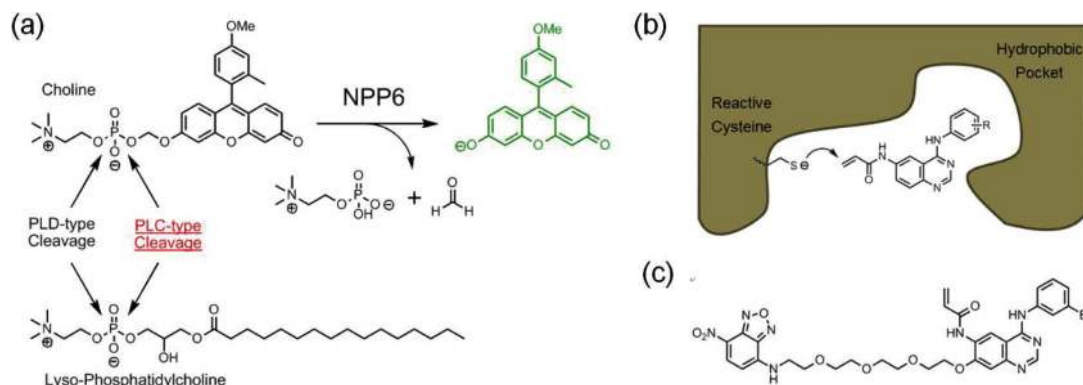


Fig. 3 (a) Design of selective fluorescent substrates for NPP6. NPP6 is known to metabolize lyso-phosphatidylcholine via a PLC-type reaction, so the substrate probe was designed to mimic the substrate structure in such a way that a PLC-type reaction would cause a fluorescence increase. (b) Design of a selective fluorescent probe for EGF receptor based on the known interaction of the aminoquinazoline scaffold with protein kinases. (c) Molecular structure of a selective labeling probe for an EGF receptor developed by optimization of interaction at the hydrophobic pocket and the site of covalent modification with cysteine in the active site.

applied to the design of fluorescent probe molecules.<sup>47a</sup> Shokat *et al.* developed a fluorescent probe that binds to the kinase active center of an EGF receptor with complete selectivity over other structurally similar kinases (Figs. 3b and 3c),<sup>47c</sup> by applying detailed knowledge of kinase structures and using a well-characterized small-molecular inhibitor scaffold.

#### 4-3 Evaluation of substrate specificity in living systems

Since thousands of enzymes are present in living systems, it often occurs that the developed fluorescent substrates are metabolized by unknown (off-target) enzymes, even when they work for proper targets in *in vitro* experiments. In those cases, the tools to correlate substrates and target enzymes (or off-targets) are required. One approach for this purpose is zymography, where non-denaturing electrophoresis is coupled to colorimetric and fluorometric substrate assays to detect active proteins on the gel.<sup>48</sup> This approach provides better separation than conventional column chromatography-based separation, and two-dimensional gel electrophoresis can provide an activity map of proteins with target activities.<sup>49</sup> So far, zymography assays for more than 400 enzymes are developed,<sup>48a</sup> but the relatively poor detection limits and consequent difficulty in spot identification in most assays remains a major problem in practical use.<sup>48b,50</sup> Recently, Komatsu *et al.* have developed the diced electrophoresis gel (DEG) assay system based on the zymography method, which employs fluorescent substrates to find and characterize the target enzymes of given fluorescent substrates,<sup>51</sup> and employed it to develop fluorescent substrates of acylamino acid releasing enzyme (APEH) with sufficient selectivity to be useful for in cellulo and *in vivo* studies. The technique is useful not only for characterization of on- and off-targets of developed fluorescent substrates, but also for discovery of novel protein functions in proteomes.

## 5 Biological Applications of Fluorescent Substrate Probes for Studying Enzymes

Small-molecular fluorescent substrate probes with the ability to report enzymatic functions are increasingly being applied to a wider range of systems. In the last chapter of this review, we describe several examples of application of fluorescent substrates

in enzymatic studies.

### 5-1 Understanding the physiological functions of enzymes

Selective fluorescent substrates can be used to study the physiological functions of the targeted enzymes, although the need for very high selectivity is an important issue here, as described above. For example, Sames *et al.* developed a series of fluorescent substrates to study the functions of oxidoreductases, such as monoamine oxidases,<sup>52</sup> AKR1C2,<sup>32</sup> 17beta-HSD10,<sup>42a</sup> and 5alpha-R1,<sup>42b</sup> at the single cell level. These probes can be used to study how the enzymatic function<sup>32,42a</sup> or the metabolic influx of physiological substrates<sup>42b</sup> is controlled in living cells under various conditions.

The key feature of live cell or living animal imaging of enzymatic activity is that it can provide information on enzymatically active regions at the tissue, cellular or even subcellular level by visualizing changes in activity. Therefore, they usually offer more detailed data than *in vitro* studies that simply monitor the enzymatic activity in the mixture.<sup>53</sup> Also, it is much more valuable to study enzymatic functions in living systems, since purification of the enzyme sometimes alters its physiological activity. An interesting example of the use of the fluorescent substrates to study the living enzymatic functions involved the fluorescent substrates of glutathione-S-transferase (GST).<sup>33b</sup> When the substrates were applied to HuCCT1 cells, a strong signal was observed in the nucleus of cells. Since the original substrate and fluorescent product did not show the particular subcellular localization, this staining pattern may reflect high activity of GST in nucleus, which, in cancer cells, might contribute to protecting cellular DNAs from damage by anticancer drugs<sup>54</sup> and oxidative stresses.<sup>55</sup> Determination of the biological significance of the observation must await further characterization of the system, but these findings could not easily have been obtained by performing biochemical studies with cell lysates.

Another example of the use of fluorescent substrates in biological studies is the identification of high aldehyde dehydrogenase (ALDH) activity in stem cells and tumor cells.<sup>37,56</sup> Since the enzyme is involved in synthesis of retinoids, this observation suggests that retinoid signaling may influence the differentiation and proliferation potential of cells, though the mechanisms involved remain to be established.<sup>56a</sup> The finding is

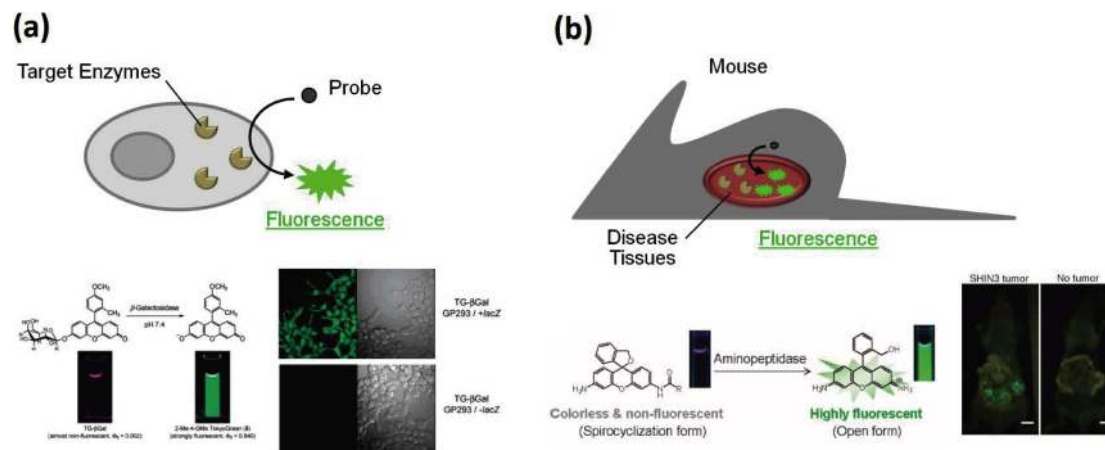


Fig. 4 (a) Principle of fluorescence imaging of enzymatic activities in living cells. (b) Principle of fluorescence imaging of living animals to detect disease tissue. Images are reprinted from Refs. 23b and 34a.

also valuable in that it offers a means to isolate stem cells from tissue extracts with the use of a fluorescence-activated cell sorter (FACS).<sup>56b</sup>

## 5.2 High-throughput screening

Small-molecular fluorogenic substrates have been used for high-throughput screening (HTS) to search large libraries of compounds for inhibitors.<sup>57</sup> Due to the increasing availability of large-scale compound libraries,<sup>57a,58</sup> HTS is of great interest to researchers in the fields of biology and medicine.<sup>59</sup> The availability of a good assay platform usually increases the chance of finding hits,<sup>33a,60</sup> and therefore fluorescent substrates, with their high sensitivity, are particularly suited to searching for enzyme inhibitors.

While genetic methods, such as overexpression and knockout, are often employed to study the functions of enzymes by modulating protein functionality, small-molecular inhibitors have specific advantages for biological studies:<sup>59b,59c</sup> (1) they are more suitable to study time- and dose-dependent changes, and (2) they have wider applicability, ranging from cells to disease models. Furthermore, they can be useful as tools in proof-of-concept (POC) studies for establishment of novel drug candidates to treat diseases. For example, Cravatt *et al.* developed a selective and potent inhibitor of MAGL,<sup>61</sup> which functions in endocannabinoid signaling,<sup>61-62</sup> tumor progression,<sup>8b,63</sup> and neuroinflammation.<sup>64</sup> *In vivo* studies with the inhibitor could connect the enzymatic functions to certain pathogenic states, which enlightened the therapeutic value of the selective inhibition of MAGL in living systems.

Fluorescent substrates are also used in phenotype-based screening.<sup>53,59a</sup> Phenotype-based assay is performed using a cell-based platform to characterize compounds that modulate cellular phenotypes, and the target is only subsequently identified. Cai *et al.* established a phenotype-based assay platform to search for inducers of apoptosis, by applying fluorescent substrates targeting caspase-3.<sup>65</sup> They discovered several compounds that induced caspase-3 activation and consequent apoptosis in living cells. While the targets of some of the hit compounds were well-characterized proteins such as tubulin,<sup>65b,66</sup> and transferrin receptors,<sup>67</sup> the screening also identified an apoptosis inducer whose main target was identified as TIP<sup>47,68</sup> which had not been known to play a role in apoptosis.

## 5.3 *In vivo* imaging and diagnosis

As a last example of applications of fluorescent substrates, they can be used for *in vivo* imaging, where a probe molecule is introduced into the circulation of living animals, and enzymatically active tissues or regions can be visualized.<sup>8h</sup> Since increased activities of some enzymes are correlated to progression of specific diseases, fluorescence imaging has the potential to be used for diagnostic purposes (Fig. 4). The idea of using enzymatic activity assays for diagnostic purposes is not new,<sup>12a,69</sup> but recent progress in fluorescence imaging systems as well as fluorescent substrates has opened up opportunities for them to be applied to living animals and even human patients.

Weissleder *et al.* developed a sensor for matrix metalloproteases (MMPs) that is suitable to visualize aggressive tumor cells,<sup>70</sup> which characteristically have high activities of MMPs. Furthermore, Urano *et al.* used a gamma-glutamyl-transpeptidase (GGT) activity probe to detect ovarian cancer cells.<sup>34a</sup> Since the elevated activity of GGT was observed for various tumor cells,<sup>71</sup> the development of a highly sensitive fluorogenic substrate has enabled the visualization and selective detection of tumor cells simply by spraying probe solution. The usefulness of these diagnostic strategies for patients remains to be fully established, but the identification of more biomarkers and development of more functional substrate probes is driving further research in this area.

## 6 Conclusions

In this review, we have presented an overview of recent progress in the design and use of fluorescent substrate probes for studying the functions of enzymes. The need to find suitable sensors to investigate enzymatic functions on a proteome-wide scale, as well as to image enzymatic activities in living cells and animals, has been the driving force behind a search for fluorescent substrate probes with extremely high specificity. Establishment of efficient methodologies for design and biological application of optimized fluorescent substrates is expected to lead to the discovery and annotation of novel enzymatic functions. Such probes are also expected to be useful for clinical diagnostic purposes, and also for monitoring the therapeutic efficacy of medicines.

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