ACCOUNTS of chemical research

Article

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Two-Photon Small Molecule Enzymatic Probes

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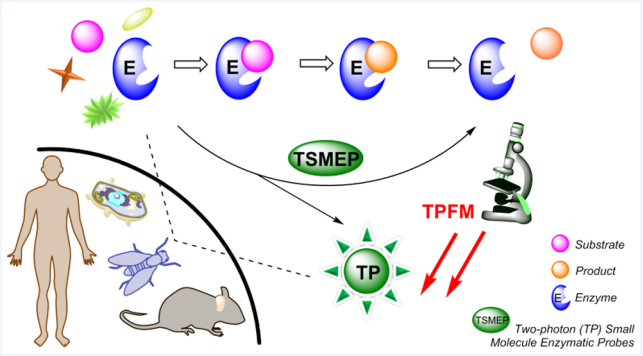
CONSPECTUS: Enzymes are essential for life, especially in the development of disease and on drug effects, but as we cannot yet directly observe the inside interactions and only partially observe biochemical outcomes, tools "translating" these processes into readable information are essential for better understanding of enzymes as well as for developing effective tools to fight against diseases. Therefore, sensitive small molecule probes suitable for direct in vivo monitoring of enzyme activities are ultimately desirable. For fulfilling this desire, two-photon small molecule enzymatic probes (TSMEPs) producing amplified fluorescent signals based on enzymatic conversion with better photophysical properties and deeper penetration in intact tissues and whole animals have been developed and demonstrated to be powerful in addressing the issues described above. Nonetheless, currently available TSMEPs only cover a small portion of enzymes despite the distinct advantages of two-photon fluorescence microscopy. In this Account, we would like to share design principles for TSMEPs as potential indicators of certain pathology-related biomarkers together with their applications in disease models to inspire more elegant work to be done in this area. Highlights will be addressed on how to equip two-photon fluorescent probes with features amenable for direct assessment of enzyme activities in complex pathological environments. We give three recent examples from our laboratory and collaborations in which TSMEPs are applied to visualize the distribution and activity of enzymes at cellular and organism levels. The first example shows that we could distinguish endogenous phosphatase activity in different organelles; the second illustrates that TSMEP is suitable for specific and sensitive detection of a potential Parkinson's disease marker (monoamine oxidase B) in a variety of biological systems from cells to patient samples, and the third identifies that TSMEPs can be applied to other enzyme families (proteases). Indeed, TSMEPs have helped to uncover new biological roles and functions of a series of enzymes; therefore, we hope to encourage more TSMEPs to be developed for diverse enzymes. Meanwhile, improvements in the TSMEP properties (such as new two-photon fluorophores with longer excitation and emission wavelengths and strategies allowing high specificity) are also indispensable for producing high-fidelity information inside biological systems. We are enthusiastic however that, with these efforts and wider applications of TSMEPs in both research studies and further clinical diagnoses, comprehensive knowledge of enzyme contributions to various physiologies will be obtained.



INTRODUCTION

Chemical probes are dedicated to illustrating biological processes as well as providing practical tools for modulating them at the interface of advanced chemistry and biology.[1] Techniques developed in this field include making the information inside the biological system "readable" with probes and enabling artificial regulation of physiological functions with newly synthesized/discovered drug candidates, which would undisputedly promote development in disease diagnosis, therapy, and prevention.[2] Within living organisms, proteins act as front-line workers that participate in virtually every process to maintain homeostasis. Over the years, our group has utilized various platforms to facilitate the study of proteins and their molecular interactions.[3−9] Specifically, we are highly interested in enzymes due to their intimate association with diseases[10] and druggability.[11,12] Comprehensive illustration of enzyme functions, together with approaches for precise detection of their activities under both healthy and pathological conditions, is fundamental for disease diagnosis and treatment.

For the precise evaluation of enzyme activities that are functionally regulated by a series of post-translational mechanisms in a dynamic manner, fluorescence imaging stands out for its ability to sensitively visualize biological targets and processes with high temporal-spatial resolution, and dramatic progress has been made over the last several decades.[13−17] A diverse array of small molecule-based fluorescent probes with desired photophysical properties and advantageous cell permeability or even organelle specificity, binding affinity, kinetics, and chemical tractability are available nowadays.[18] Among them, enzymatic probes (also known as substrate-based probes)[17,19] are competitive for bioimaging because of two

Received: November 23, 2015
Published: April 5, 2016

DOI: 10.1021/acs.accounts.5b00512
Acc. Chem. Res. 2016, 49, 626−634

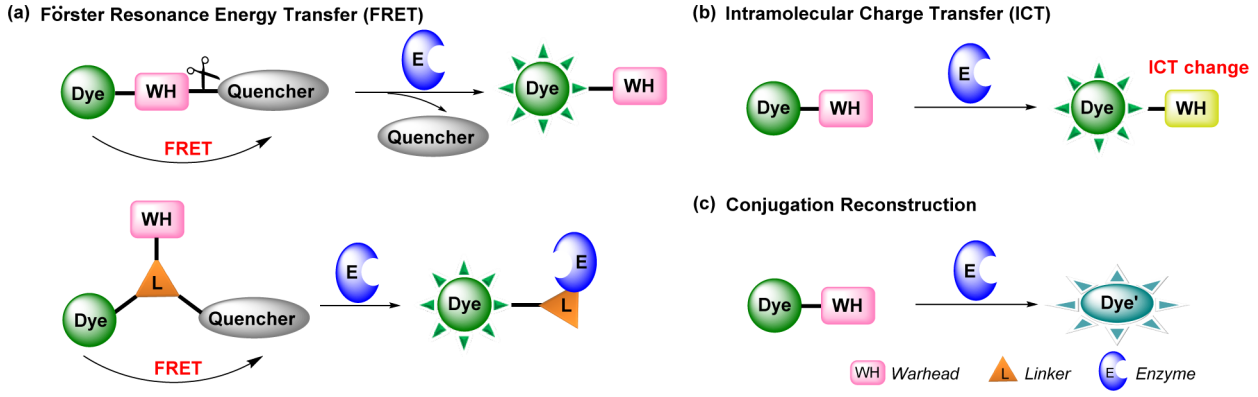


Figure 1. Representative strategies involved in two-photon small molecule enzymatic probes. (a) Enzyme-activatable probes based on FRET. (b) Substrate-like fluorogenic probes based on intramolecular charge transfer. (c) Reaction-based reconstruction of the conjugating system in the molecule leading to a fluorescence change.

imponderable advantages. One, they can mimic the intrinsic metabolic process and produce sufficient signals to be distinguished among complicated biological components, and the other, as signals originate exclusively from reactions catalyzed by the defined enzyme under native conditions, it is a direct measure of enzyme activity rather than protein expression level. It should be noted that, in most cases, what matters most in vivo is the enzyme at work, especially in the presence of endogenous inhibitors, such that the amount of protein may remain unchanged while the activity has already changed.[17]

Although an increasing number of probes have been developed to investigate various enzymes, available probes are limited for in-cell fluorescence imaging and cannot be applied to the majority of disease models constructed with tissues and animals. Fluorophores utilizing a longer wavelength for excitation can achieve deeper penetration due to reduced photon scattering (photon scattering scales as $\lambda^{-\alpha}$, where λ is the wavelength and α = 0.2−4 for biological tissues),[20] and the wavelength range of 650−1450 nm falls in the region of the spectrum with the lowest absorption in tissue (absorbance by water, melanin, proteins, and hemoglobin are high between 200 and 650 nm) and has minimal autofluorescence.[21] Remarkably, near-infrared (NIR) fluorophores activatable by one-photon have attracted significant attention in bioimaging due to inexpensive diode laser excitation. However, there are relatively few classes of NIR fluorophores readily available, including the phthalocyanines, cyanine, and squaraine dyes.[22] With constraints in the large conjugated structure, NIR fluorophores often suffer from aqueous insolubility, insufficient stability in biological systems, and low quantum yields, leaving subsequent functionalization or optimization of NIR fluorophores challenging.[23,24] Alternatively, for multiphoton dyes absorbing two or more photons simultaneously for each transition event,[25,26] an NIR laser is used to excite the fluorophore, enabling deep-tissue penetration while at the same time allowing more flexibility in the structures.[27] Moreover, because the signal (S) depends supralinearly ($S \propto I^n$, n = 2 for two-photon dyes) on the density of photons (or on the excitation intensity, I), the fluorescence is confined to the vicinity of the focal point (~1 μm^3).[27,28] This localized excitation not only provides intrinsic three-dimensional resolution in fluorescence microscopy but also lowers photodamage and photobleaching. Very recently, three-photon fluorescence microscopy has been shown to image up to 3.5 mm deep into tissue.[20] This is undoubtedly a significant step toward enlarging the depth for imaging, but collective efforts are still needed for wide applications. In particular, the development of two-photon fluorescence microscopy (TPFM) has been propelled by rapid technological advances in laser scanning microscopy, fluorescent probe synthesis, and computational three-dimensional image reconstruction.[26] Furthermore, increasing knowledge in chemistry and photophysics also enables the development of a TP fluorophore with better photophysical properties.[29]

A series of TP probes targeting biothiols, reactive oxygen species (ROS), and metal ions have already been established with outstanding advantages over one-photon (OP) probes.[28,30,31] However, TP probes for enzymes that are indispensable in homeostasis maintenance remain underdeveloped. For TPFM to be widely adopted for bioimaging of enzyme activities and to address some controversial phenomena in biology, it is essential to produce enzymatic probes that not only incorporate a TP fluorophore but also have improved properties to address the void, such as better selectivity/sensitivity and higher spatiotemporal resolution in a noninvasive manner. To highlight the "target-guided" design in two-photon small molecule enzymatic probes (TSMEPs), in this Account we will divide available TSMEPs according to their targets (1, endogenous phosphatase activity in different organelles; 2, the potential Parkinson's disease marker monoamine oxidase B; and 3, proteases) to introduce principles used for developing practical TSMEPs as well as inspirations for biological issues.

■ PRINCIPLES FOR TSMEP DESIGN

By definition, TSMEPs can be recognized by an enzyme in situ and generate amplified signals through intrinsic multiturnover enzymatic reactions.[17,19,32] Strategies employing this enzymatic conversion to "switch-on" the probe (changes in wavelength and/or intensity) usually apply one of the following mechanisms: (1) Förster resonance energy transfer (FRET) (Figure 1a),[33−36] (2) intramolecular charge transfer (ICT) (Figure 1b),[37−39] and (3) transformation of a latent fluorescent molecule into a fluorescent one upon conjugation reconstruction (Figure 1c).[40] In probes relying on FRET, a fluorophore and quencher pair are attached to each other within a certain distance via an enzyme-cleavable linker to generate a prequenched probe that restores fluorescence upon release of the quencher by a specific enzyme (Figure 1a). The ICT approach often asks for a change in electron-donating and/or

DOI: 10.1021/acs.accounts.5b00512
Acc. Chem. Res. 2016, 49, 626−634

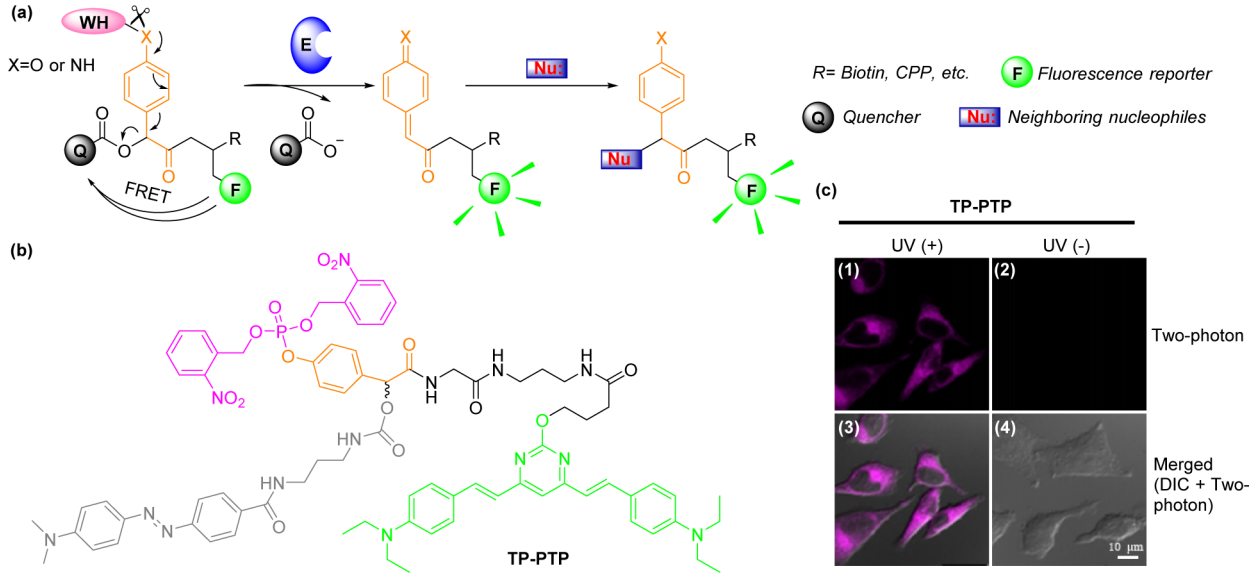


Figure 2. First TSMEP for protein tyrosine phosphatases (PTPs). (a) Overall design principle for the enzymatic probe with an anchoring property after activation. (b) Structure of **TP-PTP** in this study. (c) Two-photon images (λ_{ex} = 800 nm, taken on a Leica TCS SP5X confocal microscope system) of endogenous PTPs in HeLa cells using **TP-PTP** with (left) and without (right) UV exposure.[33]

-withdrawing groups within the molecule by enzymatic conversion (Figure 1b). A more specific method is the modulation of a molecular conjugation system based on enzyme-mediated reactions (Figure 1c), leading to a photophysical property change of the probe. Of note, photon-induced electron transfer (PeT) is another powerful method to introduce a change in fluorescence. For probes constituting a donor and an acceptor, certain chemical reactions eliminate the charge transfer between them under excitation, which restores the fluorescence. A wealth of TP probe-detecting metal ions are decorated with PeT for excellent "turn-on" efficiency.[31] Although only partially involved in a few enzymatic probes,[37] PeT is not included as a general mode here. Overall, a desirable TSMEP should undergo a detectable fluorescence change after the chemical modification catalyzed by the target enzyme.[28]

The following requirements should also be taken into consideration during probe design: (1) biocompatibility (water solubility, cell permeability, cytotoxicity, and chemical stability under native conditions), (2) sufficient in vitro potency and selectivity to indicate and fulfill its cellular or in vivo performance, (3) sensitivity, especially for proteins at low abundance, and (4) easy preparation (feasible synthetic route or readily available source). However, we can generally only build valid and objective rule-based parameters for biological systems according to those existing quality probes. When applied under physiological conditions, the quality probe discovers its own path to validation.[41]

TSMEPS FOR PHOSPHATASES

Phosphatases are a large family of enzymes that participate in signaling networks by removing the phosphate group from their substrates. Of the various types of phosphatases, protein tyrosine phosphatases (PTPs), which mainly catalyze the dephosphorylation of *p*Tyr residues in proteins, are implicated in many human diseases once under defective regulation.[42,43] There are over 100 members in the PTP family that differ in their protein structure, substrate specificity, and subcellular localization.[42]

Consequently, biological and chemical approaches capable of reporting phosphatase activities would provide invaluable insights into how these enzymes work under physiological settings and whether they are indicative during disease development. In 1939 as pioneering work in enzymatic assays, Gomori developed a method to visualize phosphatase activity in tissue sections based on the idea that a phosphate released from glycerophosphate via enzyme catalysis could be detected in terms of the formation of an insoluble salt with Ca^{2+}.[43] Thereafter, numerous strategies aimed at identifying phosphatase activities in cells were established.[44,45] However, to make the detection amenable to a wider range of biological specimens, several issues remained to be addressed, including higher sensitivity, better spatial resolution, and improved detection depth in live cells and tissues.

With these goals in mind, we came up with the first TSMEP for PTPs, **TP-PTP**, which allows signal retention and amplification without inactivation of the target enzyme for helping quinone methide chemistry.[33] The quinone methide intermediate has a distinct property that, once formed, it will diffuse from the active site at a rate competitive with the subsequent labeling process, thus causing labeling to occur away from the catalytic active site of the target enzyme.[46] By taking advantage of the reactive yet diffusible nature of this intermediate, both visualization of enzyme localization and fluorescent signal amplification to sensitively report endogenous multiturnover enzyme activity can be achieved. With a mandelic acid core, three modular components, including an enzyme substrate warhead (WH), a fluorescence reporter (F), and a quencher (Q), were strategically built around it (Figure 2a).[33] For targeting PTPs as well as to enable temporal control of imaging in live cells, a phosphate group "caged" with photolabile groups (2-nitrobenzyloxy) was incorporated. Meanwhile, a FRET pair of dabcyl quenchers and the well-known TP fluorophore, 2-hydroxy-4,6-bis(4-(diethylamino)styryl)-pyrimidine, were attached to the mandelic acid core and proximal to each other, offering a substantial quenching effect (Figure 2b). Once such low-background probes entered cells, UV irradiation can remove the caging group to present the

DOI: 10.1021/acs.accounts.5b00512
Acc. Chem. Res. 2016, 49, 626−634

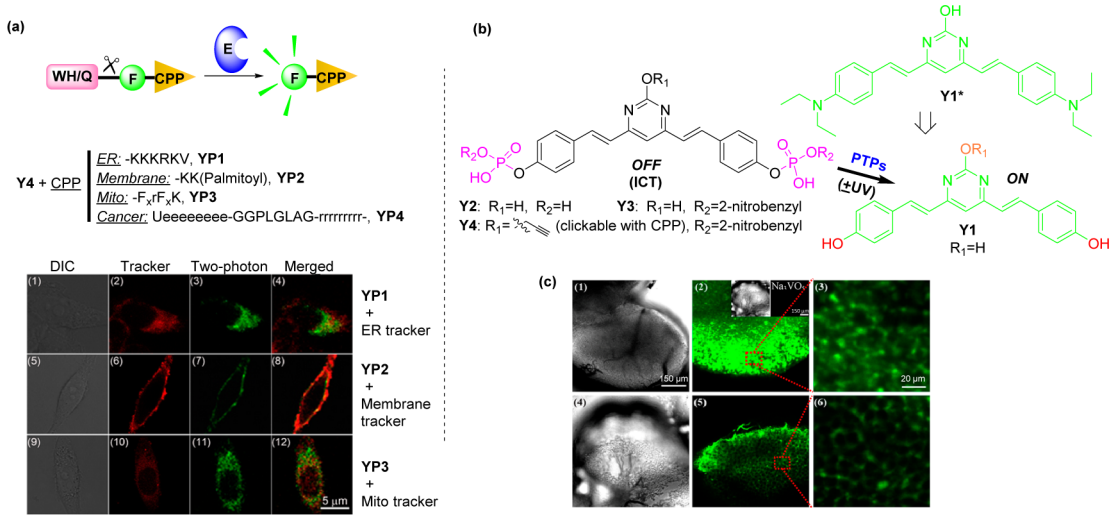


Figure 3. Organelle-specific TSMEPs for phosphatases. (a) (top) Schematic of our CPP-conjugated two-photon fluorogenic probes; (bottom) TP imaging (λ_{ex} = 800 nm) of endogenous phosphatase activities in live HeLa cells demonstrating targeted localizations with designed CPP with **YP1** in 1−4, **YP2** in 5−8, and **YP3** in 9−12. (b) Structures of reporters (**Y1**−**4**) derived from the well-known TP fluorophore (**Y1***) in this study. (c) Images of endogenous phosphatase activities in fresh brains of *Drosophila* using **Y2** and uncaged **YP2** after incubation with the probes (20 μM) for 6 h under TPFM (λ_{ex} = 800 nm) at a depth of ∼110 μm. (1−3) **Y2**, insets are images obtained from the brain treated with tyrosine phosphatase inhibitor Na_3VO_5. (4−6) Uncaged **YP2**. (3, 6) Enlarged images of the red boxes in (2) and (5) with 40× magnification.[39]

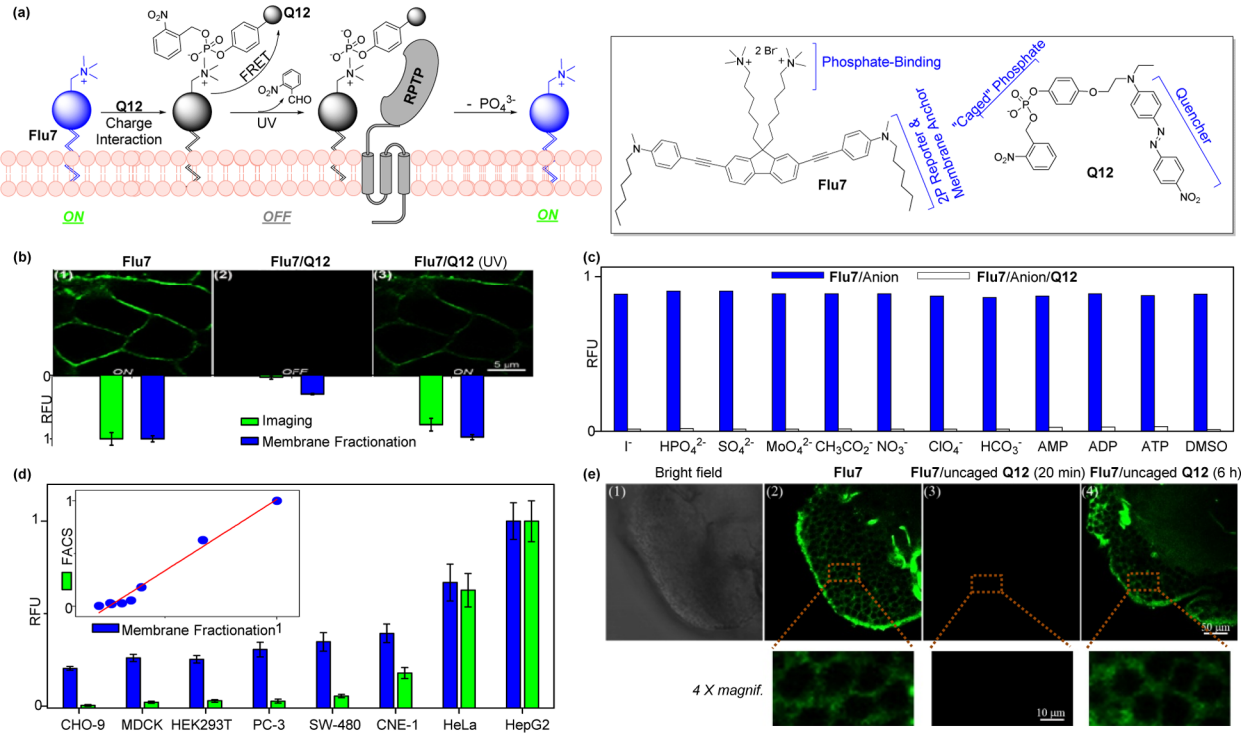


Figure 4. (a) Overall strategy of our switchable TP membrane tracer pair, **Flu7**/**Q12** (structures shown at right), which is capable of imaging membrane-associated receptor-like protein tyrosine phosphatase (RPTP) activities. (b) (top) One-photon images of HepG2 cells treated with **Flu7** (1 equiv), **Q12** (2 equiv), and UV irradiation successively; (bottom) total fluorescence intensities of the images (green) compared with those from the isolated membrane fraction of the same treated cells (blue). (c) Effect of anions (10 equiv) on **Flu7**/**Q12** fluorescence. (d) Membrane-associated RPTP activity of eight different mammalian cell lines measured by FACS (green) and membrane fractionation (blue) experiments. (inset) Correlation between these two experiments, fitted by linear regression, giving R^2 = 0.98. (e) TPFM (λ_{ex} = 770 nm) of RPTP activities in fresh brains of *Drosophila* using **Flu7**/uncaged **Q12** at a depth of ∼110 μm: (1) bright field image of brain, (2) *Drosophila* brain treated with **Flu7** (20 μM) for 2 h, (3) further incubation with uncaged **Q12** (100 μM) for 20 min, and (4) after further incubation for 6 h. (bottom) Magnification (4×) of the images in the red boxes in panels 2−4.[35]

naked phosphate group under control. In the presence of PTPs, the quencher was removed by 1,6-elimination to liberate distinct fluorescence ("turn-on") as well as to covalently label neighboring nucleophiles of the enzyme active site to indicate the localization of enzymes. With such a probe in hand, TPFM of HeLa cells was carried out and indicated strong PTP

DOI: 10.1021/acs.accounts.5b00512
Acc. Chem. Res. 2016, 49, 626−634

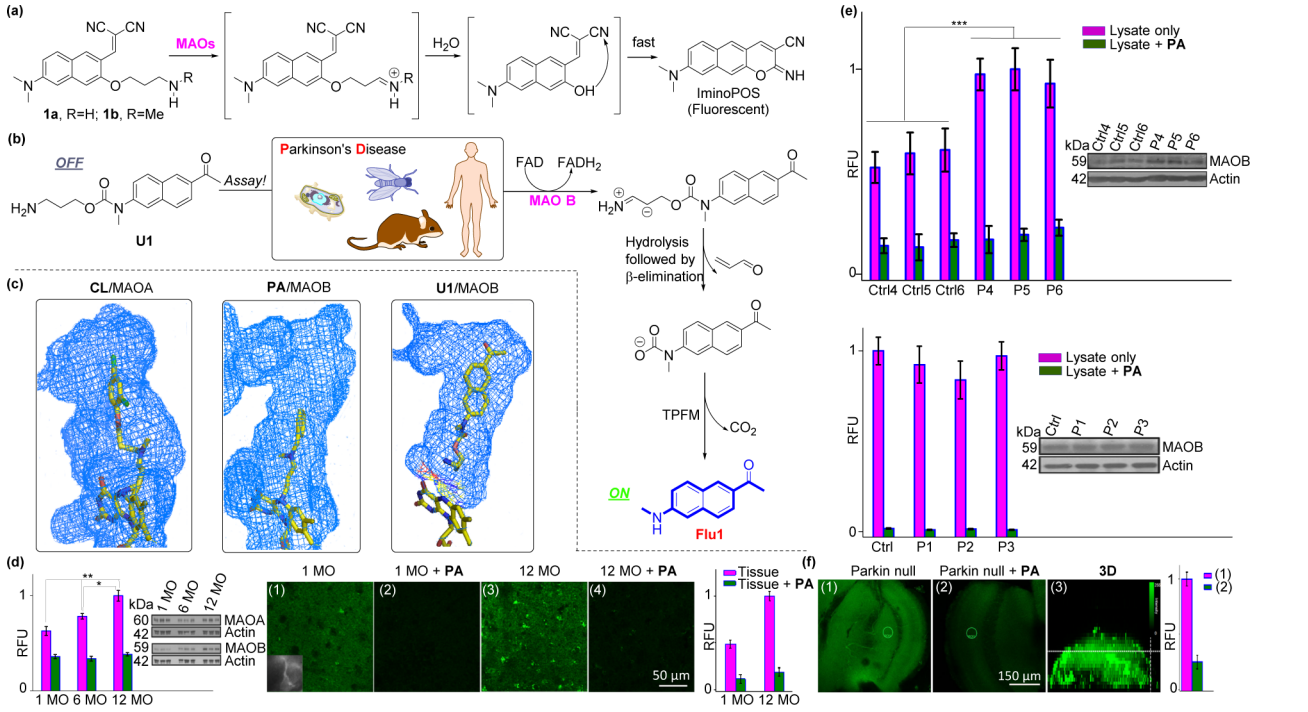


Figure 5. (a) Structure of the first TSMEP for MAOs and its "turn-on" mechanism.[40] (b) Overall strategy using **U1** for MAO-B-specific detection in live mammalian cells, *Drosophila*, mice, and B lymphocytes isolated from PD patients. (c) Docked structures of **CL** (clorgyline, MAO-A-specific inhibitor) with MAO-A (left), **PA** (pargyline, MAO-B-specific inhibitor) with MAO-B (middle), and **U1** with MAO-B (right). (d) (left) Relative fluorescence reading of **U1** (2.0 μM) + brain lysates of 1, 6, and 12 month old (1/6/12-MO) mice with and without **PA** (green/purple). (inset) WB results. (middle) TPFM (λ_{ex} = 780) of the SNpc region of fresh 1 and 12 month mouse brain slices with (1)/(3) **U1** (100 μM) and (2)/(4) **U1** (100 μM) + **PA** (200 μM) at 120 μm depth with 40× magnification. (right) Average fluorescence intensity profiles of mouse brain image panels (1−4). (e) **U1**-assisted detection of MAO-B for potential clinical diagnostics of PD in humans. (top) Relative-fluorescence reading of **U1** (2.0 μM) in proteome lysates of human B lymphocytes obtained from PD patients (P4−6) and aged-matched normal controls (Ctrl4−6). (inset) WB results of the corresponding samples. (bottom) Relative-fluorescence reading of **U1** (2.0 μM) in lysates prepared from fibroblasts of PD patients (P1−3) and the corresponding age-matched controls (Ctrl). (inset) WB results of the corresponding samples. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$; Student's t test, two-tailed in (d) and (e). (f) (left) TPFM (λ_{ex} = 780 nm) of fresh brain slices of 20 day old parkin-null *Drosophila* with (1) **U1** (100 μM) and (2) **U1** (100 μM) + **PA** (200 μM) at a depth of 100 μm with 40× magnification. (3) Reconstructed 3D TP image of (1). (right) Average fluorescence intensity profiles of (1)/(2) at the indicated position.[37]

activities in cytosol (Figure 2c). Meanwhile, additional tags, such as biotin for further protein profiling studies or cell-penetrating peptides (CPPs) to facilitate cellular entrance, can be introduced when needed. Such an activatable TSMEP was introduced for the first time in the detection of enzyme activities; more significantly, the modular design here can be applied to other hydrolytic enzymes (e.g., all four major classes of proteases) as well.

One of the driving issues in the development of potent small molecule probes for high-resolution imaging is their very fast diffusion. In addition to molecules described above using a highly reactive quinone methide intermediate to "fix" the fluorescent adduct near the site of the reaction, a "targeting" moiety is another choice. It can be conjugated to the probe for specific delivery and enhanced subcellular retention with less interference to protein activities than probes containing reactive groups. To this end, fluorogenic probes employing ICT and furnished with targeting moieties were developed (Figure 3a).[39] Substitution of 4-diethylamino in **Y1*** to 4-hydroxy gave **Y1** with a TP action cross-section ($\delta\cdot\Phi$ = 82 GM) comparable to those of other common TP fluorophores (Figure 3b).[47] Once attached by the electron-withdrawing phosphate group (phosphatase-responsive warhead) at two phenolic groups (**Y2**), a significantly reduced conjugated π-electron system led to much weaker red-shifted fluorescence emission of the probe as the "OFF" state. For sufficient delivery of the probes without premature interaction with phosphatases outside the desired organelle (phosphatase activity was ubiquitously present at most intracellular spaces and organelles), the phosphate groups were protected by photolabile moieties (i.e., 2-nitrobenzyl group in **Y3**/**Y4**). Moreover, the targeting groups terminated with an azide can easily be assembled to the alkyne derivative **Y4** for detection of localized enzyme activities. Four representative CPPs specific for endoplasmic reticulum (ER, linker-KKKRKV), plasma membrane (linker-KK (Palmitoyl)), mitochondria (linker-F_xrF_xK), and cancer cells (Ueeeeeeee-GGPLGLAG-rrrrrrrrr-linker; cleavable by matrix metalloproteases that are overexpressed on the surface of numerous cancer cells, including HeLa cells) were conjugated to give **YP1−4**, respectively (Figure 3a). After the probes accumulated at the targeted location, the protecting group was removed by quick UV irradiation to interact with PTPs therein. Encouragingly, the "turn-on" fluorescence signal linked by CPPs can be spatially stable afterward, which benefits imaging (Figure 3a, bottom). As expected, this novel TP system succeeded in imaging endogenous phosphatase activities in *Drosophila* brains with a detection depth over 100 μm (Figure 3c).

One potential drawback of the former design is that the localized signal may not all come from PTPs within the targeted organelle, as the premature fluorophore "turned-on"

DOI: 10.1021/acs.accounts.5b00512
Acc. Chem. Res. 2016, 49, 626−634

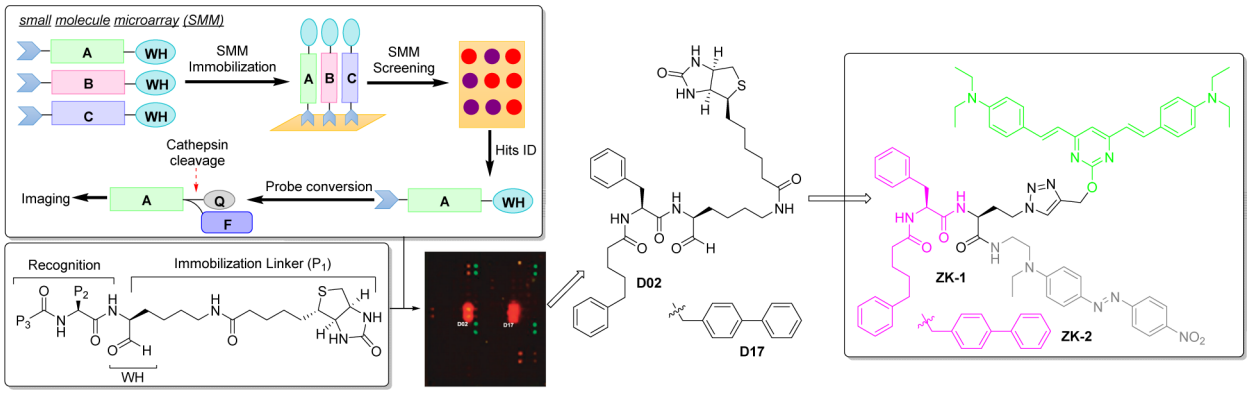


Figure 6. Overall strategy of the small molecule microarray (SMM)-guided, high-throughput discovery of cell permeable TSMEPs (**ZK-1** and **ZK-2**) for imaging of endogenous cathepsin L.[36]

by PTPs from somewhere else can also be driven to the organelle by the CPPs. For addressing this, a novel "ON/OFF/ON" system was proposed to ensure the exclusive detection of membrane-associated, receptor-like protein tyrosine phosphatases (RPTPs) (Figure 4a).[35] First, **Flu7**, which contains the π-conjugated fluorene moiety modified with two 6-carbon aliphatic chains for membrane anchoring, could serve as a TP membrane tracer as the "ON" state. The other two hydrophobic tails in **Flu7** were specifically capped with positively charged quaternary ammonium groups. Therefore, a negatively charged pairing partner, **Q12**, consisting of a fluorescence quencher (Disperse Red 1) and a "photo-caged" phosphate group, can bind to **Flu7** through electrostatic interactions, thus effectively quenching the fluorescence through intermolecular FRET ("OFF" state). Subsequently, upon UV irradiation, the deprotected phosphate can undergo enzymatic dephosphorylation by membrane-associated RPTPs. Finally, the dephosphorylated **Q12** would dissociate from **Flu7** to restore its fluorescence ("ON" state). In this way, the recovered signal only came from the membrane-associated RPTPs, and it was further confirmed by a membrane fraction assay (Figure 4b). Interestingly, common biological anions showed little effect on **Flu7**/**Q12** fluorescence (Figure 4c), indicating the association of **Flu7**/**Q12** was driven not only by electrostatic interactions but also by other noncovalent interactions like $\pi-\pi$ stacking and hydrophobic interactions. For compatible live-cell imaging and strong restriction to membrane-associated PTPs, elevated activities of RPTP in cancer cell lines were differentiated by this probe from the normal cells (Figure 4d), consistent with reports that increased endogenous RPTP activity is responsive to tumorigenesis in numerous cells and tissues.[42] Ultimately, the utility of this system in TPFM was established successfully when imaging RPTP activity deep inside live *Drosophila* brains (Figure 4e). Similarly, the FRET strategy was also successfully applied by Xing et al. for real-time imaging of a cell surface-associated proteolytic enzyme (i.e., furin-like convertase) activities, where the fluorophore and quencher were linked by a cleavable peptide.[48]

TSMEP FOR MONOAMINE OXIDASE B

Monoamine oxidases (MAOs) are integral proteins of the outer mitochondrial membrane that catalyze the oxidative deamination of monoamine neurotransmitters, including noradrenaline, adrenaline, and dopamine, along with the release of hydrogen peroxide (H_2O_2).[49,50] With the ability to terminate physiological actions of these neurotransmitters, the expression level and enzymatic activities of MAOs are crucial to the nervous system. Two isoforms of this enzyme (MAO-A/-B) have been identified in humans, which differ in distribution, substrate selectivity, and inhibitor sensitivity, thus having distinct clinical significances.[51,52] One example is the age-related neurodegenerative disorder Parkinson's disease (PD), such that the expression of MAO-B but not MAO-A increases with age, and its activity is significantly enhanced in the brains of PD patients.[53] At present, there is no convenient diagnostic biomarker for PD, and in the clinic, PD is mainly diagnosed based on symptoms and neuroimaging tests, such as dopamine-based positron emission-computed tomography (PET) imaging with exclusively high cost. With evidence suggesting MAO-B to be a potential PD marker but not conclusive yet, it is urgent to look for more practical means in MAO-B detection to explore the unknowns and facilitate clinical diagnosis of PD.

The majority of known fluorescence sensing systems for MAOs are based on one-photon fluorophores;[54−57] only Ahn and co-workers developed compounds **1a** and **1b** as TP probes for MAOs, for the first time, by applying the known reaction-based sensing scheme (Figure 5a).[40] A few of them are selective for MAO-A/-B,[57] but they have not been well-investigated in central neuron disease models or clinic samples. To fill up this gap, **U1**, a TSMEP suitable for specific and sensitive detection of MAO-B activities in a variety of biological systems including established PD models, was developed recently (Figure 5b).[37] **U1** employed 2-methylamino-6-acetylnaphthalene (acedan, **Flu1**) as the TP reporter and 3-aminopropyl carbamate as the MAO responsive unit. Compared with MAO-A, the active site of MAO-B is longer and narrower and consists of an "entrance" cavity (290 Å^3) and a hydrophobic "substrate" cavity (490 Å^3) occupied by the redox-active isoalloxazine ring of FAD coenzyme at the distal end; thus, **U1** can fit into the "substrate" cavity of MAO-B snugly for distinct specificity (Figure 5c). Initially, the electron-poor carbamate bond in **U1** quenched the intrinsic fluorescence of acedan through a PeT and ICT mix effect. Upon MAO-B-catalyzed oxidation of the amino group to aldehyde, a propionaldehyde moiety is released spontaneously by β-elimination followed by self-immolative discharge of CO_2 to liberate the highly fluorescent **Flu1**. With such outstanding contrast between background and signal, **U1** was indeed a probe suitable for continuous monitoring of MAO-B activity in both live cells and tissues by TPFM. As shown in Figure 5d and f, **U1** can unambiguously report the difference of MAO-B activities in PD models from the control ones, including age-associated increases in MAO-B expression

DOI: 10.1021/acs.accounts.5b00512
Acc. Chem. Res. 2016, 49, 626−634

(Figure 5d) and parkin-related regulation of MAO-B (Figure 5f). Intriguingly, elevated MAO-B activities were detected in human B lymphocytes from PD patients by **U1**, suggesting that the MAO-B level in peripheral blood cells is a potential biomarker for rapid diagnosis of PD (Figure 5e). Given the high MAO-B selectivity provided by acedan, which also works as a built-in fluorophore, a dual-purpose activity-based probe capable of in situ profiling and live-cell imaging of MAO-B was developed based on the key features of both **U1** and the proteome profiling-enabled probes as the first example to accomplish what all previously reported MAO-B imaging probes failed to do.[58]

TSMEPS FOR PROTEASES

Among various enzymes, proteases are particularly attractive not only because they are large in number but also due to their roles in controlling numerous biological processes as signaling molecules.[59] According to their intrinsic property of proteolysis, probes aimed at them often contain a peptide derived from their endogenous substrates for recognition. However, overlap in substrates between different proteases, especially those in the same family, impedes their applications as precise sensors. Efforts have been made to improve selectivity among certain protease subfamilies through screening for the optimal peptide sequence.[60] For achieving this, high-throughput screening (HTS) platforms are desirable. Our group demonstrated a convenient system by using small molecule microarray (SMM) as an HTS platform for the successful discovery of two potent inhibitors of cathepsin L, which were converted strategically to the corresponding TSMEPs (Figure 6).[36]

CONCLUSIONS

Two-photon probes are indeed welcome in preclinic investigations and are promising for in vivo imaging with advanced technologies, such as microendoscopy,[61,62] as information closer to real physical conditions can be obtained with direct operation on tissue samples or whole animals. Therefore, the TSMEPs described above should be applied to explore information that may be overlooked with traditional methods. Meanwhile, it is essential to enlarge the inventory of TSMEPs to cover more medically interesting enzymes (mainly hydrolases, oxidoreductases, and transferases as the most common enzymatic drug targets)[11] because TSMEPs available now only correspond to less than a dozen enzymes (i.e., phosphatases, monoamine oxidase, and proteases). For this to be accomplished, TP fluorophores with better photophysical properties (e.g., brighter and longer excitation and emission wavelengths, superior stability) that are amenable to multicolor imaging are highly desirable. Meanwhile, long-standing issues in enzyme detection, such as sensitivity, selectivity, and biocompatibility (e.g., permeability, less invasive for biological applications) should be considered in novel TSMEP design. Attention should also be given to moving away from the description of an individual protein, instead viewing various interactions as a whole to understand the signaling network. Therefore, real-time as well as multicolor imaging allowing simultaneous detection of several proteins will be helpful. Furthermore, quantification is becoming more appreciated in fluorescence imaging of enzymes to distinguish between normal and disease state; thereby, TSMEPs with internal inference are very promising. We are enthusiastic that, with more effort delivered to this field, next-generation TSMEPs possessing greater capabilities will soon be developed to meet these specific needs.

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Funding

Grants from the Singapore National Medical Research Council (CBRG/0038/2013), the Ministry of Education (MOE2013-T2-1-048), Fundamental Studies of Perovskite Solar Cells (2015CB932200), National Natural Science Foundation of China (61505076), and Natural Science Foundation of Jiangsu Province (BK20140951) are acknowledged.

Notes

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