

Recent biomedical advances enabled by HaloTag technology

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Key words: HaloTag, Site-specific labeling, Biomolecule interaction, Molecular imaging, Positron emission tomography

Abstract: The knowledge of interactions among functional proteins helps researchers understand disease mechanisms and design potential strategies for treatment. As a general approach, the fluorescent and affinity tags were employed for exploring this field by labeling the Protein of Interest (POI). However, the autofluorescence and weak binding strength significantly reduce the accuracy and specificity of these tags. Conversely, HaloTag, a novel self-labeling enzyme (SLE) tag, could quickly form a covalent bond with its ligand, enabling fast and specific labeling of POI. These desirable features greatly increase the accuracy and specificity, making the HaloTag a valuable system for various applications ranging from imaging to immobilization of POI. Notably, the HaloTag technique has already been successfully employed in a series of studies with excellent efficiency. In this review, we summarize the development of HaloTag and recent advanced investigations associated with HaloTag, including *in vitro* imaging (e.g., POI imaging, cellular condition monitoring, microorganism imaging, system development), *in vivo* imaging, biomolecule immobilization (e.g., POI collection, protein/nuclear acid interaction and protein structure analysis), targeted degradation (e.g., L-AdPROM), and more. We also present a systematic discussion regarding the future direction and challenges of the HaloTag technique.

Introduction

A series of biological processes (e.g., development of diseases) are kept happening in the body, which was triggered by complex interactions between biomacromolecule (e.g., protein-protein or protein-nucleic acid). Therefore, a comprehensive understanding of these interactions would reveal the mechanisms of certain diseases and advance the strategies for early diagnoses and therapies. Given that cellular proteins are highly dynamic, genetic modification is the best approach for investigating a protein of interest (POI) at active status. Generally, the labeling of POI via affinity tag (e.g., His-Tag) or fluorescent protein (e.g., GFP) allows an intracellular tracking or immobilization of POI. However, several drawbacks cannot be avoided entirely. For instance, the impurity of harvested proteins could be caused by unspecific binding (e.g., His-Tag) or a decrease in the brightness of fluorescence, etc. These disadvantages potentially restrain their applications in studies that require high accuracy (e.g., miRNA-protein interaction or POI tracking studies).

In the last two decades, a series of self-labeling enzymes, also called self-labeling enzymes (SLEs), had been successfully developed. Three prominent examples are the CLIP-tag/SNAP-tag (19.4 kDa), ACPTag (9 kDa) and HaloTag (33 kDa) (Table 1). These SLEs share several features, including relatively small size, high stability, and fast-reacting kinetics, allowing fused POI to be labeled by tag-specific substrates with extremely high specificity and efficiency. More importantly, the functionalization of interactive substrates allows fusion proteins to be immobilized on a solid phase, monitored via molecular imaging (e.g., fluorescence, luminescence or radioactive isotope, etc.) or gifted capacities (e.g., redox-response or ion-activated imaging). As such, the POI or the interacting biomolecules on the POI could be specifically studied via the functional substrates that covalently bind to POI for various purposes, even high-resolution imaging of cellular ion influx. In the current review, we summarize the developments of HaloTag technology, in particular, recent applications of HaloTag for POI tracking (e.g., *in vitro* and *in vivo* imaging), immobilization (e.g., protein-reacting mRNA analysis and protein structure study), targeted degradation (e.g., chloroalkane-containing proteolysis targeting chimeric (HaloPROTACs)) and other applications (e.g., differentiation of stem cell), is highlighted (Scheme 1). In addition, the

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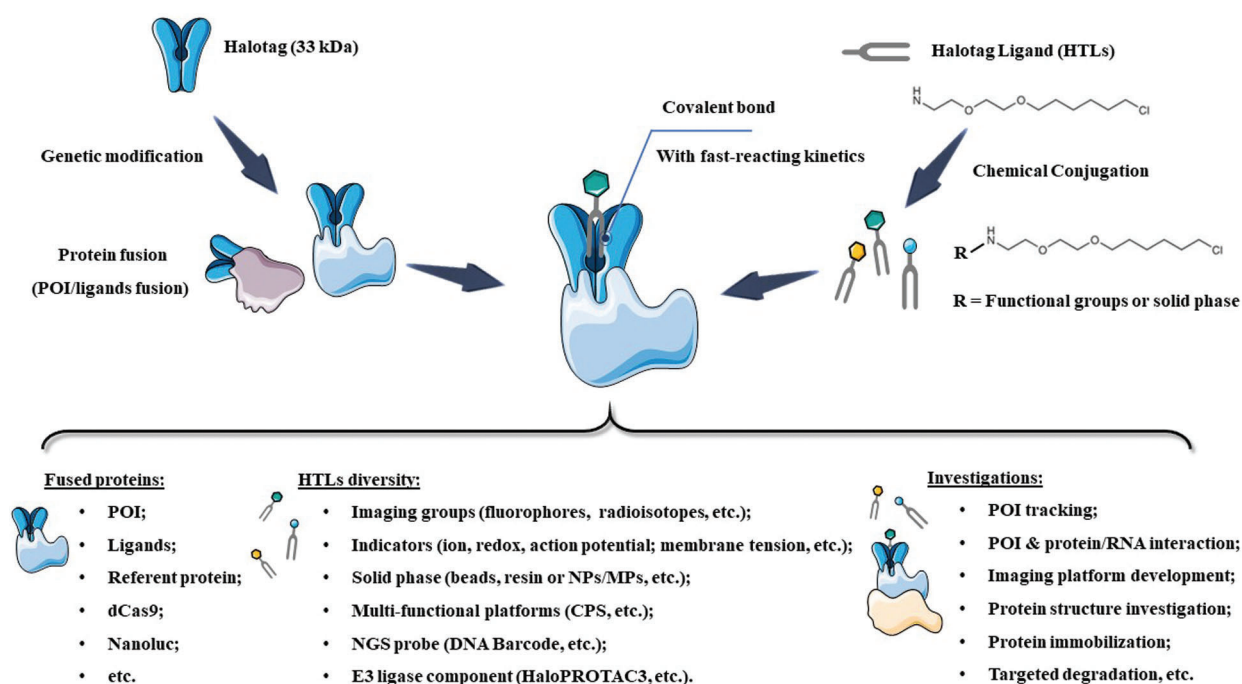
Received: 16 July 2021; Accepted: 02 December 2021



TABLE 1
Comparison of HaloTag among other general tags

Tags	Size (kDa)	Functions	Major applications	Advantages	Drawbacks
His-Tag	0.2–1.6	Affinity	Protein purification	Small size	Non-specific binding
GST-Tag	~26	Affinity/ Solubility	Protein purification	High affinity, Improvement of solubility	Large size; Contamination of HSP in mammal cells
Strep-Tags	0.244	Affinity	Protein purification	High affinity	Contamination of biotinylated proteins in mammal cells
EGFP	32.7	Fluorescence	Fluorescence imaging	Brightness	Large size; Influence of dimer formation
mCherry	28.8	Fluorescence	Fluorescence imaging	Quick formation than other mRFP	Large size
ACP-Tag	9	Site-specific binding or labeling	Imaging of POI	Small size	Impenetrable substrate (surface labeling only)
SNAP/ CLIP-Tag	19.4		Targeted studies of POI via imaging, drug delivery and immobilization, etc.	Small size	Relative lower labeling kinetics ($2.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)
HaloTag	33		Targeted studies of POI via imaging, drug delivery, immobilization, structure study and targeted degradation, etc.	Rapid labelling kinetics ($2.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$); High stability;	Large size

Note: GST: Glutathione-S-transferase; EGFP: Enhanced green fluorescence protein; HSP: Heat-shock proteins; mRFP: Monomeric red fluorescent protein.



SCHEME 1. Scheme of HaloTag system, as well as the recent designs and investigations. NPs: nanoparticles; MPs: micro-sized particles; NGS: Next-Generation Sequencing; POI: Protein of Interest; CPS: Cell-penetrating streptavidin.

prospects and challenges for future developments of HaloTag are systemically discussed.

A Brief History of HaloTag Technology

HaloTag (33 kDa) is modified from the bacterial enzyme haloalkane dehalogenase, with its Phe272 residue mutated to His272. The mutation of this residue results in a loss of its

ability to perform hydrolysis. As a result, an alkyl-enzyme intermediate can be formed between the HaloTag and its ligand without further hydrolysis, leading to the formation of a covalent bond. Although HaloTag is an exogenous protein, it does not interfere with an organism's normal physiological metabolism (Naested *et al.*, 1999). By recruiting different designs of ligands, HaloTag is applicable in various directions of research and investigation. For

example, the folding of HaloTag can be directly monitored via the change of fluorescent intensity from a conjugated ligand, which may serve as a desirable platform to study the POI folding procedures during translation or other high-complexity conditions (Samelson *et al.*, 2018).

Since Promega Corporation developed HaloTag in 2005 (Los *et al.*, 2005), this technique has received growing attention from scientists worldwide. Within one year, the high efficiency of HaloTag system was investigated and verified by several research groups (Lang *et al.*, 2006; Zhang *et al.*, 2006). For instance, the Rao group successfully used HaloTag technology to induce a site-specific modification of bioluminescent protein on quantum dots (QDs) (Zhang *et al.*, 2006). As-prepared QDs were labeled with HaloTag-fused renilla luciferase fusion protein via surface conjugation of HaloTag ligand (HTL), which could be lightened up by bioluminescence resonance energy transfer (BRET) after the attendance of the substrate, coelenterazine. Although HaloTag was only designed for applications in mammalian cells initially, researchers ingeniously employed this platform in a series of different research fields. In the following decade, HaloTag technique was comprehensively studied and applied in studies including protein interactions (Löchte *et al.*, 2014), localization of POI within cells (Lee *et al.*, 2010; Strauch *et al.*, 2011; Liu *et al.*, 2012), and functional HaloTag ligand (HTL) developments (Raina *et al.*, 2014; Neklesa *et al.*, 2011). In particular, advanced HTL such as HyT36 and HyT13 generated from the Crews group has paved new ways of observing certain mechanisms (e.g., endoplasmic reticulum (ER) regulation) inside cells (Raina *et al.*, 2014; Neklesa *et al.*, 2011). More specifically, these HTL could induce the destabilization of HaloTag-fused POI on ER and cause a resolvable ER stress, revealing the relation between unfolded protein response (UPR) and estrogen-mediated ER stress. With these fundamental research bases, HaloTag gradually became a powerful tool that has been widely applied in various basic and applied researches (Schlichthaerle *et al.*, 2019; Li *et al.*, 2020). In the following sections, we will summarize these latest and novel investigations associated with HaloTag strategy (Table 2).

Recent Biomedical Applications of HaloTag Technology

In vitro imaging

Imaging POI in living or fixed cells could offer vital information for understanding their biochemical functions inside protein networks (Ohno *et al.*, 2014). In support of HaloTag system, POI fused with HaloTag could be effectively labeled by HTL. As such, a series of approaches for tracking POI in cell or micro-organs, monitoring cellular conditions and developing advanced platforms for therapy or imaging could be achieved via different conjugations of functional groups on HTL, such as dyes (e.g., rhodamines, carbopyronines, Si-rhodamine, Alexa Fluor) (Takahashi *et al.*, 2019; Frei *et al.*, 2019; López-Andarias *et al.*, 2020; Thevathasan *et al.*, 2019).

POI imaging

As the general approach for POI imaging, HaloTag is directly fused with POI (e.g., G Protein-coupled receptors and receptor tyrosine kinases, etc.), enabling site-specific labeling

via HTL and imaging groups conjugated (e.g., fluorophores) (Berki *et al.*, 2019; Butkevich *et al.*, 2016; Lesiak *et al.*, 2020; Peach *et al.*, 2021). For instance, a HaloTag fused serum response factor (SRF) was designed by Hipp *et al.* (2019) to investigate the interaction between SRF and chromatin in fibroblast and primary neuron cells. Significantly, the fusion of HaloTag did not induce any interference on SRF's location and functions. With the assistance of HTL-fluorophore (TMR and silicone rhodamine), chromatin residence times of SRF could be accurately detected (up to 1 min) and defined as three regimes via single-molecule living imaging. Similarly, Frei *et al.* (2019) successfully visualized HaloTag-fused POI (TOMM20) on the outer mitochondrial membrane via single-molecule localization microscopy (SMLM) (Fig. 1A). Specifically, novel photoactivatable silicon rhodamine (PA-SiR) with super spectroscopic properties was developed and conjugated on HTL. Under UV irradiation (Fig. 1B), PA-SiR bound to TOMM20 could be protonated and visualized via SMLM for over 65 s in living cells (Figs. 1C–1E). In addition, Huet-Calderwood *et al.* (2017) reported a complex platform for imaging integrin *in vitro* by integrating various fluorescent tags (e.g., GFP, pHluorin, or HaloTag) with $\beta 1$ integrin (ecto- $\beta 1$) via HaloTag technique. In particular, the highly specific ecto-Halo $\beta 1$ was able to track and analyze $\beta 1$ integrin with detailed spatiotemporal dissection for up to 1 h, showing the high accuracy of HaloTag strategy.

Unlike most studies recruiting conventional fluorophores, a light-independent/luminescent imaging strategy was developed by Chang *et al.* (2019) (Figs. 2A and 2B). In this system, HaloTag was fused with nanoluc that could behave as a bioluminescence resonance energy transfer (BRET) donor. Once the furimazine was presented, nanoluc will be lighted up and excite HTL-DEAC450 (coumarin-based dye), with fast kinetics ($t_{1/2} < 120$ s) (Fig. 2A). More importantly, after photolysis, the uncaging ibrutinib from HTL-DEAC450 induced efficient therapy on HeLa and SKBR3 cells (Fig. 2B).

However, the imaging of single POI only provides limited information, which is insufficient for studying interactions between biomacromolecules or high-accuracy researches. Thus, innovative dual-tracking approaches have been developed for identifying the interactions between POI & POI or POI & RNA (Sato *et al.*, 2017; Yoon *et al.*, 2016). For example, the Singer group introduced a modular design for tracking a POI and its mRNA simultaneously in neurons from genetic-modified mice. The β -actin mRNA was encoded with 24 tandem MS2 aptamers at 3'-UTR, while HaloTag was fused to tag β -actin. With the attendance of endogenous-expressed stdMCP-stdGFP (MS2 capsid protein (MCP) and GFP fusion protein) and fluorescent HTL (i.e., JF549/JF646), β -actin mRNA and β -actin could be visualized simultaneously with high resolution.

Instead of dual-labeling, incorporating multiple HTL (with different physiochemical features) and HaloTag-fused POI could also help some investigations on biological progress. As one representative application, Takahashi *et al.* (2018, 2019) tactfully utilized HaloTag-fused microtubule-associated protein one light chain 3 (HT-LC3) and different HTL (cell-penetrated/unpenetrated) to visualize the

TABLE 2

Recent advanced investigations associated with HaloTag technology

Application	HaloTag fused protein	HTL modification	Fusion/testing model	Design of assay	Highlights	Reference
BRET-induced FL-Imaging/ drug delivery	Nanoluc	Coumarin- ibrutinib	SKBR3 cells	Luminescence induced BRET and hydrolysis	Novel design of luciferase Induced BRET and bioluminescence	(Chang <i>et al.</i> , 2019)
FL-Imaging	LC3	AF488p- MILs, TMR- MPLs	U-2 OS cells	HTLs (MILs and MPLs) completion for halotag- LCs labeling	Discover ESCRT mechanism of phagophore closure	(Takahashi <i>et al.</i> , 2019)
FL-Imaging	5HT ₆	BAPTA- JF549 derivatives	hrPE/neuron cells	Ca ²⁺ chelation induced FL emission	Develop sensitive indicator for Ca ²⁺ detection	(Deo <i>et al.</i> , 2019)
FL-Imaging	PDGFR/DAF	PEG-RhoVR	Rat neuron cells	RhoVR induced FL emission	Develop sensor for high speed imaging in brain	(Deal <i>et al.</i> , 2020)
FL-Imaging	OM/CM	JF646	<i>E. coli</i>	Difference of HTL- labeling area between intact or broken <i>E. coli</i>	Evaluate anti-bacterial effects of various AMP	(Yang and Weisshaar, 2018)
FL-Imaging /drug delivery	Mitochondria	Streptavidin/ CPS	Hela cells	4 binding sites on CPS that could carry drug or FL	Develop CPS platform for multiple applications	(López- Andarias <i>et al.</i> , 2020)
FL-Imaging	Nup96	Cy5/AF647	U2OS cells	Specific labelling of HTL on nuclear pore	Set a reference as SMLM control	(Thevathasan <i>et al.</i> , 2019)
SPECT-Imaging	N/A	DTPA- ¹¹¹ In	LS174T tumor- bearing mice	Labeling of HaloTag & Antibody via click chemistry	Set 2-step SPECT imaging for tumor detection	(Knight <i>et al.</i> , 2017)
FL-Imaging	PSD95	SiR	PSD95-HaloTag knocked in mice	Specific labelling of HTL to postsynaptic membrane (PSD95)	<i>In-vivo</i> brain (PSD95) STED imaging development	(Masch <i>et al.</i> , 2018)
Proteins immobilization	SPIN1	Beads/TMR and 505 start	293FRT cells lysis	Pre-imaging of interaction via FRET; Solid phase immobilization	SNAP & HaloTag two- step purification of bound POI	(Liu <i>et al.</i> , 2020)
Protein (enzyme) immobilization	PpBFD, L476Q and LbADH	Beads (Resins)	<i>E. coli</i>	Solid phase immobilization via HTL	Effect purification of enzyme (with sustained biocatalyst)	(Döbber <i>et al.</i> , 2018)
NGS & Proteins immobilization	Autoantibody (DGS)	DNA barcodes	Human serum	Protein detection via DNA barcode reading (PCR and NGS)	High sensitivity (10 ⁴ times wider range than traditional ELISA)	(Yazaki <i>et al.</i> , 2020)
Protein & miRNA immobilization	Ago2	Beads/TMR	Developing embryos, mESCs, adult tissues	UV mediated protein & miRNA crosslinking; RNA sequencing	Detection of miRNA in mice with/without tumor	(Li <i>et al.</i> , 2020)
Magnetic tweezers	Titin-TEV	Glass	HaloTag-TEV titin mice	TEV-based isolation; protein length study under magnetic force	Develop magnetic tweezers for analyzing folding mechanisms	(Rivas-Pardo <i>et al.</i> , 2020)

Note: FL: Fluorescence; SPECT: Single-photon emission computerized tomography; ESCRT: Endosomal sorting complex required for transport; NGS: Next-generation sequencing; OM/CM: Outer and cytoplasmic membrane; SiR: Silicon rhodamine; STED: Stimulated emission depletion; SMLM: Single-molecule localization microscopy; POI: Protein of interest; CPS: Cell-penetrating streptavidin; AMPs: Antimicrobial peptides; FRET: Förster resonance energy transfer; PeT: Photo-induced electron transfer.

procedure of autophagosome formation. In addition, Takahashi *et al.* also recruited HaloTag system for investigating endosomal sorting complexes that were required in the formation of transport-III (ESCRT-III) component (CHMP2A) and ESCRT-I subunit (VPS37A). The genome screen on CRISPR library and HT-LCs platform successfully identify these complexes as critical factors for phagophore completion.

Monitoring cellular status

Physicochemical conditions are of vital importance for maintaining cellular functions and responses. Using HaloTag, investigators have developed a series of site-specific sensors for detecting the alteration of ion level (e.g., Mg²⁺, Na⁺, Zn²⁺, Ca²⁺) (Deo *et al.*, 2019; Gruskos *et al.*, 2016; Matsui *et al.*, 2017; Taguchi *et al.*, 2018; Zastrow *et al.*, 2020), redox change (Jiang *et al.*, 2019; Parvez *et al.*, 2016),

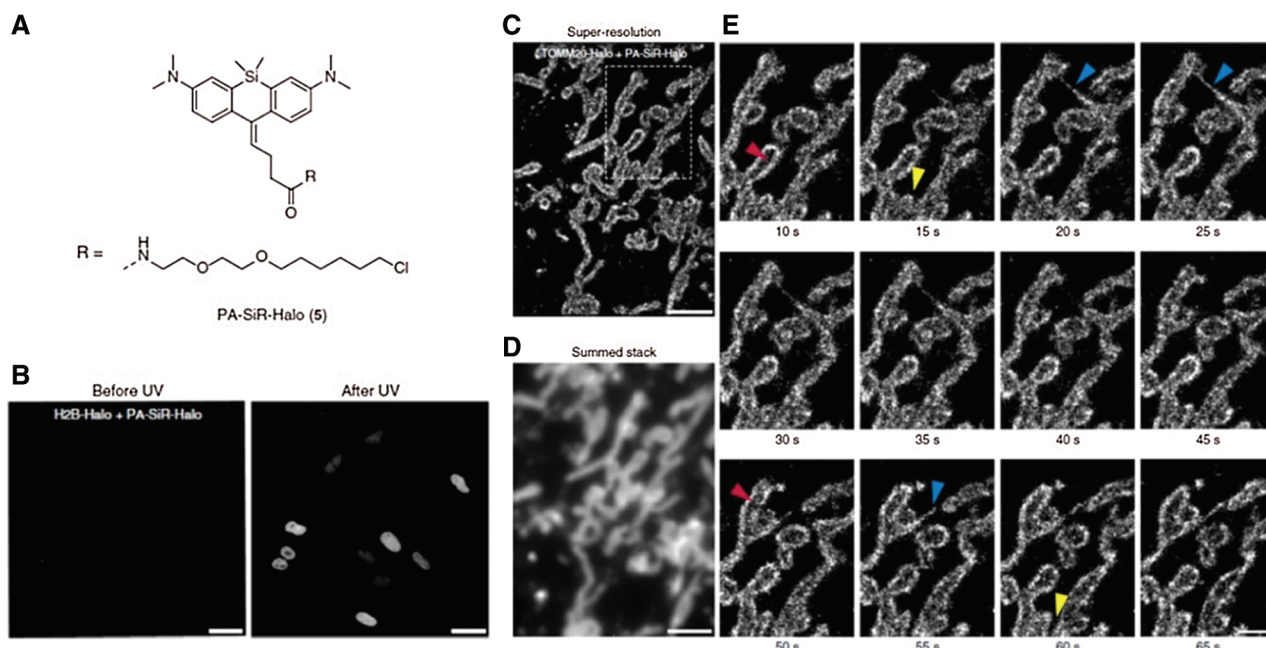


FIGURE 1. (A) Chemical structure of PA-SiR-Halo. (B) Living cell imaging on U-2 OS cells expressing H2B-Halo (nucleus) stained with PA-SiR-Halo before and after UV irradiation via SMLM; Scale bar, 40 μm . (C) Super-resolved SMLM image of outer mitochondrial membrane (TOMM20-Halo) after PA-SiR-Halo staining. (D) Summed stack image for mimicking diffraction-limited image. (E) Series of images at different time points. Arrowheads indicate hollow mitochondria (due to the TOMM20 localized to the outer membrane) (red), thin tubules formed by highly dynamic mitochondria neighboring mitochondria (blue) and disconnect (fission) in other areas (yellow); Scale bar, 1 μm . Reproduced with permission from [Frei et al. \(2019\)](#).

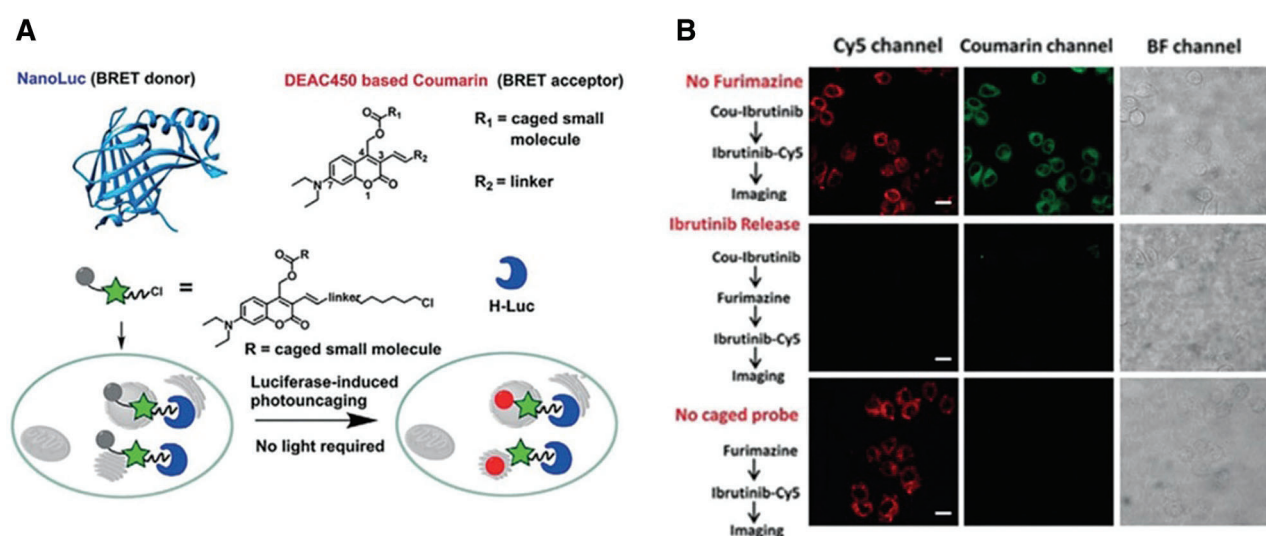


FIGURE 2. (A) Scheme of BRET-induced photolysis to release small molecule, bioluminescence. Briefly, the BRET will happen between nanoluc (fused with HaloTag) and DEAC450 (conjugated with HTLs) when furimazine encounters with nanoluc. (B) The release of ibuprofen mediated by coumarin BRET photo-uncaging in live SKBR3 cell. Scale bar = 20 μm . Reproduced with permission from [Chang et al. \(2019\)](#).

action potential ([Jiang et al., 2019](#); [Parvez et al., 2016](#)) and cell membrane tension ([Strakova et al., 2020](#)). In consideration that sodium and calcium ions are two crucial secondary messengers associated with most biochemical activities, [Deo et al. \(2019\)](#) synthesized novel Ca^{2+} indicators that were conjugated with HTLs. Among a series of HTL-indicators, the 12_{AM} synthesized could generate a site-specific and bright signal that was approximately 7.8 times that produced by jRGECO1a (verified Ca^{2+} indicator) in hippocampal neurons. More importantly, the Ca^{2+} influx of an entire organelle (primary cilium, HaloTag-fused 5HT_6)

was clearly visualized by the HTL-indicator (13_{AM}) after stimulation of o-nitrophenyl-EGTAAM.

Although action potential is strongly related to calcium ion influx, the kinetics of voltage gating is too fast to be monitored by general Ca^{2+} indicators. Inspired by photo-induced electron transfer (PeT), [Dear et al. \(2020\)](#) reported voltage-sensitive rhodamine (RhoVR-Halos) that was conjugated to HTL with polyethyleneglycol (PEG) as the linker ([Figs. 3A and 3B](#)). RhoVR-Halos effectively bound to HaloTag-fused membrane ([Fig. 3A](#)), showing high sensitivities in HEK cells ($34\% \pm 2\% \Delta F/F$ per 100 mV),

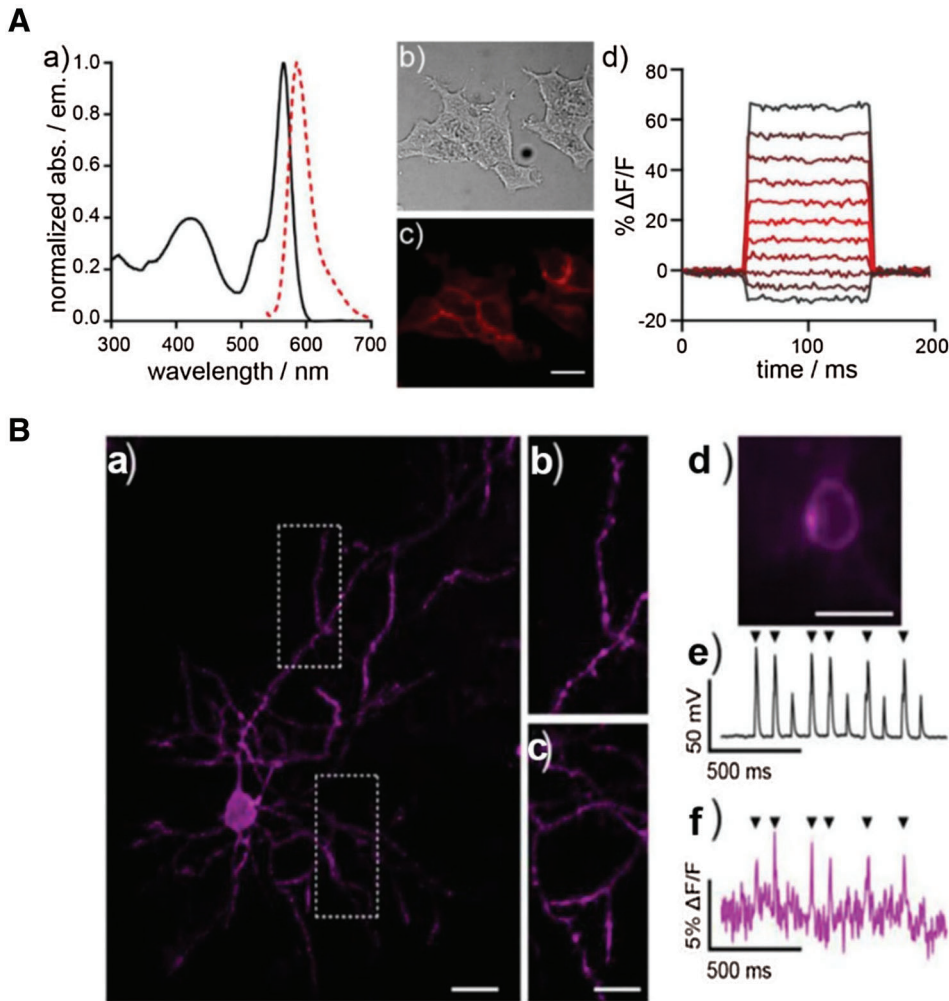


FIGURE 3. (A) The characterization of voltage sensor RhoVR. (a) Normalized spectra of absorption and emission at the concentration of 500 nM; (b) Bright field and (c) fluorescence imaging of HEK293T cells with staining of RhoVR. (d) Plot of fractional change in terms of fluorescence ($\Delta F/F$) vs. time; (B) Imaging of brain slice isolated from a mouse expressing pDisplay-HaloTag via two-photon microscopy after staining of RhoVR-Halos. (a) RhoVR fluorescence imaging of brain slice and (b) & (c) enlarged images; Scale bar is 10 μm . (d) Widefield fluorescence image of RhoVR-Halos fluorescence in a cortical neuron expressing HaloTag-pDisplay; Scale bar is 20 μm . (e) Plot of voltage vs. time for the neuron during current injection to evoke action potentials. (f) Plot of $\Delta F/F$ vs. time for the same neuron. Arrows indicate evoked spike. Reproduced with permission from Deal et al. (2020).

cultured rat neurons ($6.7\% \pm 0.2\%$ $\Delta F/F$ per spike) and neurons in slice ($4.3\% \pm 0.3\%$ per spike). Furthermore, a dual-functional indicator consisting of RhoVR-Halos and GCaMP (Ca^{2+} indicator) was successfully developed to monitor V_m and Ca^{2+} influx simultaneously (Fig. 3B).

Given the crucial role of redox in cellular homeostasis, several sensors have been investigated for surveying redox status (Jiang et al., 2019; Parvez et al., 2016). Parvez et al. (2016) creatively established a redox-monitoring technique named Targetable Reactive Electrophiles and Oxidants (T-REX) by recruiting a lipid-derived signaling electrophiles endogenous carrier (4-hydroxynonenal (HNE)) and HaloTag platform. This universal platform was capable of monitoring dynamic redox change around HaloTag-labeled POI by photo-mediated uncaging ($t_{1/2} < 1\text{--}2$ min) (HTL-PreHNE), exhibiting potential as a powerful tool for screening redox-related targets. At the same time, Jiang et al. (2019) succeeded in synthesizing a smart HTL-RealThiol (HLT-RT) that could achieve a site-specific GSH detection (nucleus and cytosol). Specifically, HLT-RT was able to detect GSH in HeLa cells and primary hepatocytes via ratiometric fluorescence (blue/green channel fluorescence). The employment of this advanced reversible probe (HLT-RT) in T-REX may efficiently prolong the timespan for organelle or cell imaging, achieving a lifetime survey in a real-time manner.

Interestingly, Strakova et al. (2020) developed a sensor to probe membrane tension, which is now a commercial product, Flipper-TR. This sensor functions by generating red-shift fluorescence and extending its lifetime by changing “twist” form to “planar” structure. Once the tagged cell membrane became tense, the signal could be precisely excited and the change of lifetime was recorded, with an increase of about 0.37 to 0.27 ns and -0.02 ns for rhodamine (control).

Imaging of microorganisms

Similarly, a comprehensive understanding of microbes would effectively promote the development of interrelated diagnosis and therapy as well. In recent years, several HaloTag-assisted studies have been reported for investigating microbes, including bacterial imaging (Barlag et al., 2016; Lepore et al., 2019; Spencer et al., 2018), evaluation of therapeutic agents (Yang and Weisshaar, 2018), and virus tracking (Liu et al., 2016). As an illustration, Liu et al. (2016) used HaloTag to label VP26, the smallest capsid protein on the pseudorabies virus (PrV). Eventually, they succeeded in visualizing two generations (parental and progeny) of PrV inside cells with HTLs (TMR and R110), which offers valuable information for understanding the whole lifespan of PrV inside cells. Additionally, Yang and Weisshaar (2018) created genetically engineered *E. coli* expressing HaloTag in outer and

cytoplasmic membranes (OM/CM). This model was subsequently used to evaluate therapeutic effects of anti-bacterial agents (MM63:CHx37, AMP LL-37, and AMP CM15) according to the permeabilization of HTL-JF646 in treated *E. coli*.

Imaging/delivery system development

The desirable specificity of HaloTag efficiently ensures its accuracy and stability, potentially extending its application for developing novel imaging/delivery systems, such as enabling specific POI as the high-fidelity control for advanced microscope development. Similar to the previously described technique for anti-bacterial evaluation, several assessment platforms based on HaloTag technique were reported too. For example, the chloroalkane penetration assay (CAPA) was designed as a high-throughput assessment for evaluating the cell penetration of therapeutic agents under various conditions (e.g., variations in temperature, time and serum), assisted by HTL and an organelle-specific organelle-specific HaloTag (Peraro *et al.*, 2018).

It is notable that a creative streptavidin-based platform and a series of small molecules have been synthesized for multiple applications (López-Andarias *et al.*, 2020). This chemical group, cell-penetrating streptavidin (CPS), consists of 4 binding sites for loading or releasing cargos via desthiobiotin/biotin interaction or desthiobiotin-biotin exchange (Fig. 4A). With the support of chloroalkane penetration assay (CAPA) and HaloTag system, López-Andarias *et al.* (2020) were able to screen the best combination (CPS carrying four benzopolysulfanes, BPS₄) for cytosolic delivery from numerous CPS-loaded drug candidates, potentially indicating the high efficiency of HaloTag technology (Fig. 4B).

Meanwhile, HaloTag has been involved in the microscope system or related dye developments as well. The method for single-molecule localization microscopy (SLSM, also named photoactivated localization microscopy, PALM) calibration is remarkably restrained due to the insufficient control data. To remedy this, Thevathasan *et al.* (2019) used genetic modification to produce various Nup96 proteins, the major component in nucleic pore complex (NPC) fused with a series of tags (e.g., HaloTag). With high-resolution imaging quality and super-fidelity supported by HaloTag, these engineering cells were successfully used as references for SLSM development, including resolution calibration, labeling efficiencies quantification, and molecular counting. In the same year, an innovative imaging strategy for DNA point accumulation in nanoscale topography (DNA-PAINT) was also developed (Schlichthaerle *et al.*, 2019). Schlichthaerle *et al.* used dye-labeled docking DNA to induce transient labeling on Nup96-Halo-HLT-DNA. These strategies resulted in desirable SLSM images with excellent resolution. Such measured distance on adjacent Y-complexes of NUP96 proteins even reached 12 nm, which was inconsistent with electron microscope (EM) imaging. Additionally, the Xiao group successfully applied a rhodamine spirolactam (Rh-Gly) probe for PALM application in support of HaloTag (Ye *et al.*, 2019). By incorporating a carboxyl group at a site near the lactam group, newly-synthesized Rh-Gly exhibited enhanced brightness, improved signal-to-noise ratio, desirable temporal resolution (10 s), and excellent accuracy for localization

(about 25 nm). Based on the site-specific labeling via HaloTag technique, a super-resolution PALM imaging of HaloTag-fused H2B in HeLa and MCF-7 cells was successfully achieved. It is reasonable to predict that more HaloTag-based techniques will be involved in and effectively promote the development of advanced imaging systems.

In vivo imaging

Due to its high specificity, the HaloTag system demonstrates great potential for various *in vivo* imaging technologies, such as PET, which is highly sensitive, quantitative, and clinically-wide used (Gan *et al.*, 2020; Jadvar *et al.*, 2007; ten Hove *et al.*, 2021). Notably, the Cai group has successfully applied this strategy for various PET-imaging via Cu-64 labeling HTLs (Hong *et al.*, 2011, 2013). More specifically, a string of NOTA-conjugated HTL constructs was synthesized and evaluated for their PET imaging efficiencies (labeled Cu-64) in 4T1-HaloTag-ECS cells or mice bearing 4T1-HaloTag-ECS tumors (Figs. 5A and 5B). Among all, the ligand ⁶⁴Cu-NOTA-HTL2G-L demonstrated the best capability for targeting 4T1-HaloTag-ECS tumor with 4.0 ± 0.2% ID/g at 6 h post injection (Fig. 5C), clearly illustrating the feasibility of the HaloTag system for *in vivo* tracking of a POI or targeting cells. Additionally, the Cornelissen lab further demonstrated the viability of HaloTag in ImmunoPET imaging, reporting HaloTag-labeled PET tracers for tumor pre-targeted ImmunoPET imaging (Knight *et al.*, 2015, 2017).

Furthermore, Knight *et al.* (2015, 2017) mediated a conjugation of HaloTag to anti-HER2 (Trastuzumab) and TAG-72 (CC49) antibodies via a Lys-to-Lys reaction. SPECT Imaging with ¹¹¹In-HLT-3 showed that tumor uptakes were about 2.8 ± 1.0% ID/g (Trastuzumab) and 3.2 ± 0.3 ID/g (CC49) at 4 h post injection, while internalizations of tracers in the control group were about 2.1 ± 0.4% or 2.1 ± 0.3 ID/g. Notably, these pre-targeted PET imaging techniques offered relatively lower imaging efficiency, which may be caused by the physiochemical features of HTL, especially the limited circulation time.

Given these, Masch *et al.* (2018) administrated a fluorescent dye conjugated HTL (SiR-HTL) via intracranial injection on the surface of brain to enhance targeting efficiency. High-resolution imaging of the PSD95-HaloTag was achieved via STED (stimulated emission depletion) nanoscopy, with the smallest measured widths (FWHM) being about 50–60 nm. This shows that novel designs of HTLs or tracers with HaloTags would increase the feasibility of HaloTag for translating from basic research to clinical trials.

Biomolecule Immobilization via HaloTag

POI purification and collection

Besides, HaloTag system also demonstrates desirable efficiency in the purification and harvest of POI, which has been widely evaluated. The fusion of HaloTag allows POIs to form a covalent bond and be extracted (from bacterial or cell lysis) by the HTL immobilized on solid phase (beads, resin or nano/macroparticles) via a rapid, specific, and strong binding procedure (Döbber *et al.*, 2018; Friedman Ohana *et al.*, 2016; Liu *et al.*, 2020; Norris *et al.*, 2020; Peschke *et al.*, 2017; Kilpatrick *et al.*, 2017). Notably, this highly efficient HaloTag approach shows a great advantage

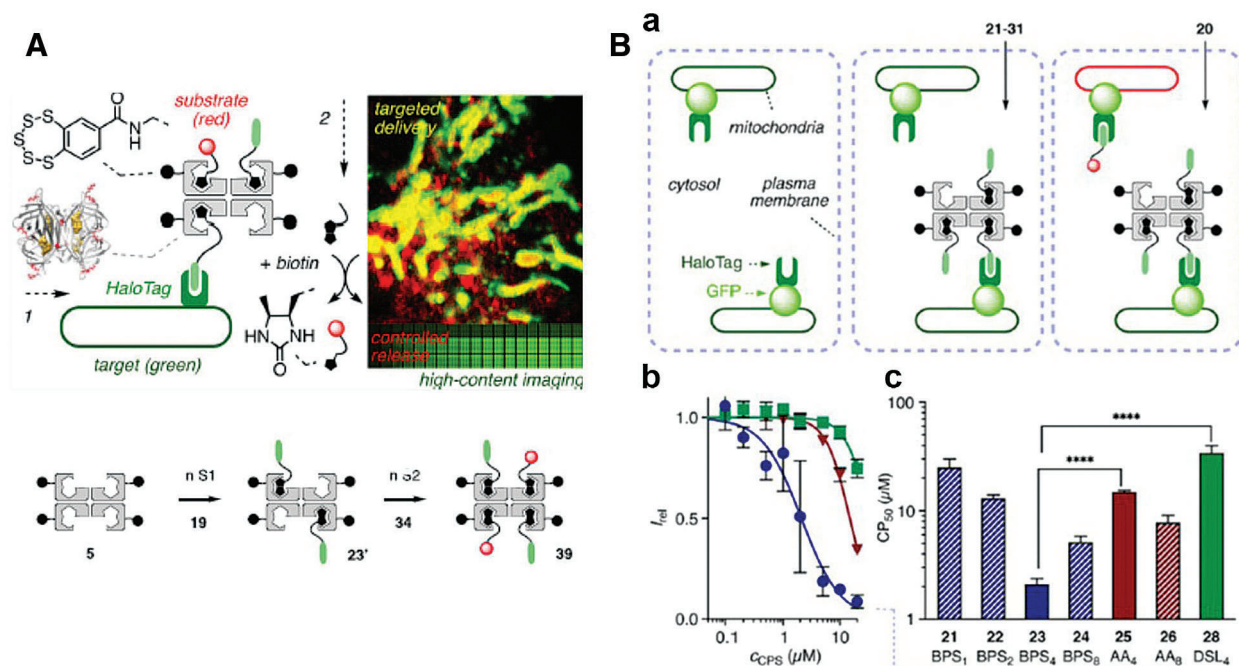


FIGURE 4. (A) Scheme of cell-penetrating streptavidin (CPS) system. The CPS is able to load different functional groups via streptavidin and biotin interaction, which could be integrated with HaloTag technique for site-specific drug delivery or imaging. (B) (a) Schematic representation of CAPA. (b) HC-CAPA dose-response curves for different CPS loading cargos, 23 (Circle), 25 (Triangle), and 28 (Square). (c) The CP_{50} values of various CPS loading cargos 21–26 and 28. Reproduced with permission from López-Andarias *et al.* (2020).

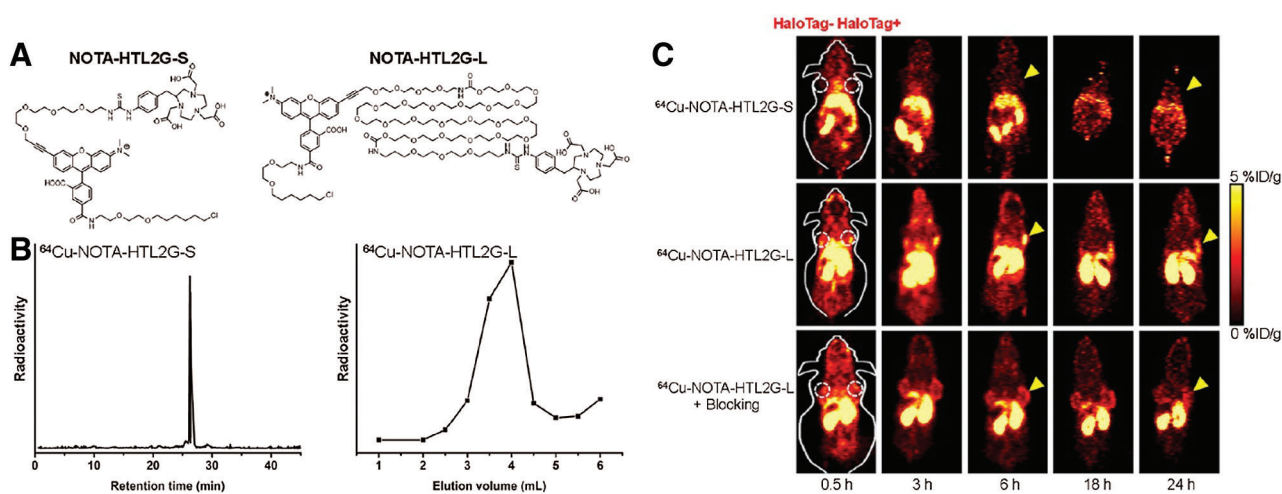


FIGURE 5. (A) Chemical structures of NOTA-HTL2G-S and NOTA-HTL2G-L. (B) HPLC of ^{64}Cu -NOTA-HTL2G-S and size exclusion chromatography of ^{64}Cu -NOTA-HTL2G-L. (C) Serial PET images of mice bearing both 4T1 (left) and 4T1-HaloTag-ECS (right) tumors at different time points post-injection of ^{64}Cu -NOTA-HTL2G-S, ^{64}Cu -NOTA-HTL2G-L, or ^{64}Cu -NOTA-HTL2G-L with blockage (N = 4). Reproduced with permission from Hong *et al.* (2013).

in enzyme purification instead of chromatographic purification (Erkelenz *et al.*, 2011; Döbber *et al.*, 2018). For instance, two HaloTag fusion enzymes, HaloTag-PpBFD L476Q and HaloTag-LbADH, were effectively harvested via a simple commercial immobilization procedure (HaloLink™ Resin) in a recent study (Döbber *et al.*, 2018). These enzymes demonstrated desirable abilities for single or cascade biocatalysts, with a high space-time yield of 1850 g/L/d for first-step catalysts and 38 g/L/d for second-step catalysts. Notably, the immobilized HaloTag-LbADH was still active even two weeks later.

Meanwhile, an advanced system named Serial Capture Affinity Purification (SCAP) was reported by Liu *et al.* (2020).

The interaction between two POI (Spindlin1 and SPINDOC incorporated SNAP-tag or HaloTag, respectively) was systemically examined via Förster resonance energy transfer (FRET), fluorescence cross-correlation spectroscopy quantitative imaging and SCAP cross-linking mass spectrometry (MS). The signal was generated via FRET between TMRDirect™ HTL (HaloTag) and 505-Start SNAP ligand (SNAP) during the interaction. Then, after the efficient enrichment of the protein complex by serial purifications (SNAP and HaloTag capture magnetic beads), this complex was obtained with high quality. In combination with MS, the spindlin1 and SPINDOC complex could be studied comprehensively, suggesting a structure consisting of a ratio of 2:1.

Most importantly, this specific-purifying technology also fulfills the high demands of clinical examinations in terms of specificity and quality. Studies were extended to patients' serum assays for screening autoantibodies, such as p53, GTF2B and Desmoglein 3 (Garranzo-Asensio *et al.*, 2016, 2020; Yazaki *et al.*, 2020). For instance, Barderas group recruited HaloTag fused POI (e.g., p53 and GTF2B) for detecting autoantibodies in serum via ELISA (Garranzo-Asensio *et al.*, 2016, 2020). In a different way, Yazaki *et al.* (2020) creatively induced a unique DNA single-strand into a POI-HaloTag complex and applied the next-generation sequencing (NGS) as an analytical procedure for serum assays (Fig. 6A). The 1:1 conjugated oligonucleotide and NGS ensure the accuracy of the HaloTag system and dramatically enhance the sensitivity of detection, showing over 10^4 times wider dynamic range than ELISA (Fig. 6B).

Investigation of protein and nucleic acid interaction

Numerous interactions between proteins and nucleic acids, ranging from transcription factors & ORF to RNA-binding proteins (RBPs) & RNA, have been involved in most physiological activities (e.g., regulation of gene expression and development). Although these complicated interacting networks are intricate for most technologies, the HaloTag system shows desirable potential in exploring such interactions. After the attachment of a HaloTag, some studies have shown that POI and interacting molecules (e.g., RNA or proteins) could be immobilized for further analysis (Brannan *et al.*, 2016; Li *et al.*, 2020). For example, Brannan *et al.* (2016) developed an RBP classifying strategy termed SONAR, and subsequently used it to discover new RBPs attaching to the same RNA as a HaloTag fused RBP. With MS and enhanced crosslinking-immunoprecipitation (CLIP), 12 nuclear and cytoplasmic RBPs were successfully identified and investigated. Meanwhile, a Halo-Enhanced Ago2 Pull-Down (HEAP) was designed for identifying micro-RNA (miRNA) targets (Li *et al.*, 2020). Specifically, the harvest and purity of miRNA-mRNA-Ago2-HaloTag were greatly ensured by the HaloTag system *in vivo* via CLIP. This strategy was further evaluated

and validated in mouse embryonic stem cells (mESCs), developing embryos, and adult tissues. After HaloTag-mediated harvest, a strong signal of miR-200bc-3p was observed in 3'UTR *Zeb2*, (EMT regulator) in autochthonous mouse-bearing human brain and lung cancers, which was in agreement with previous studies (Si *et al.*, 2017).

Protein structure analysis

Additionally, HaloTag anchoring techniques create an excellent opportunity to understand protein structure more intimately. Although the force-clamp atomic force microscope (AFM) has demonstrated its ability to assist in studying protein dynamics, the issue of mechanical drift strongly limits its sensitivity and measuring duration. To remedy this, the Fernández group creatively generated novel magnetic tweezers by combining the HaloTag approach and active correction of the focal drift. The As-designed system monitored protein L folding dynamics at low force (0–60 pN) for up to two weeks, enabling the long-term study of protein folding and misfolding, like chronic traumatic brain damage. Subsequently, Popa *et al.* further generated a mice model carrying a HaloTag-TEV genetic cassette for examining protein dynamics (titin) *in vivo* under various forces (Rivas-Pardo *et al.*, 2020). As expected, the POI, HaloTag-TEV-titin could be specifically severed and immobilized via TEV protease and HTL. Under force generated by magnetic tweezers, Popa *et al.* (2016) found the titin (I-band region) domains remained in the unfolded state and created 41.5 ZJ of mechanical work for refolding when the pulling force was less than 10 pN. It can be expected that this HaloTag-based strategy would promote the exploration of biomechanical functions associated with various proteins.

Targeted Degradation via HaloTag

Affinity-directed protein missile (AdPROM) system is a functional protein complex that could trigger the degradation of specific proteins. In combination with PROTACs and HaloTag, Buckley *et al.* (2015) developed a novel AdPROM,

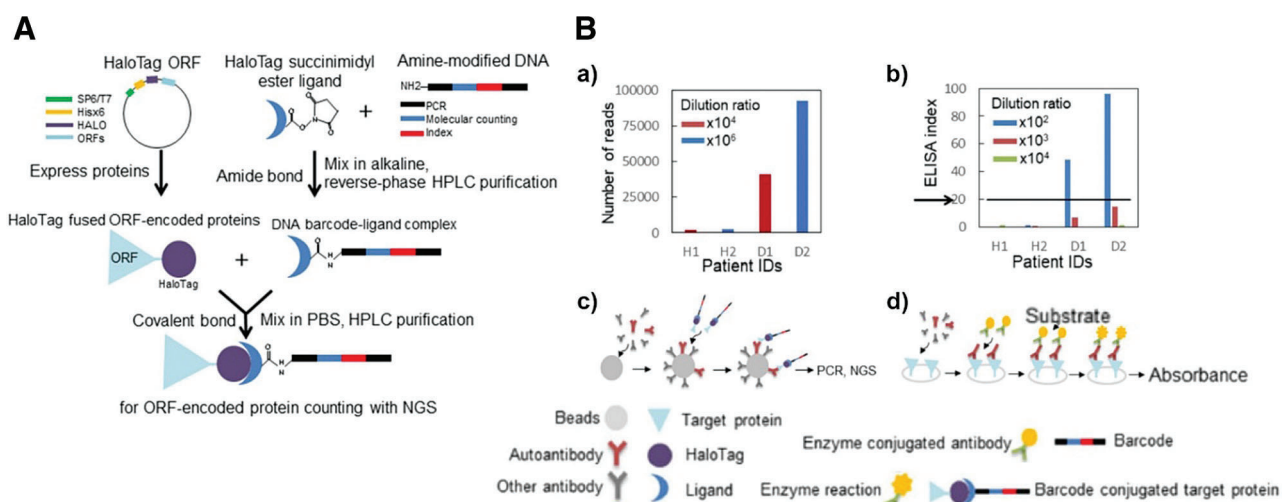


FIGURE 6. (A) Schematic diagram of the barcoding assay via HaloTag protein. The HTL is linked with specific amino-modified oligonucleotides as DNA barcodes. (B) Autoimmune antibody in patient serum detected via (a,c) barcoding assay or (b,d) conventional ELISA. Reproduced with permission from Yazaki *et al.* (2020).

named HaloPROTACs. As critical factors, the interaction of HTL and fused HaloTag directly induce the ubiquitylation on POI via the bridging of E3 ubiquitin ligase (Caine *et al.*, 2020; Schiedel *et al.*, 2020; Simpson *et al.*, 2020). Based on HaloPROTACs, Simpson *et al.* (2020) successfully synthesized a ligand-inducible AdPROM (L-AdPROM) system by combining two site-specific binding strategies, HaloTag and von Hippel-Lindau (VHL). The applied ligand consisted both of HTL and VHL moiety, which could induce the specific binding between VHL-fused AdPROM and FLAG-aGFP_{6M} (anti-GFP nanobody)-Halo. Subsequently, the degradation of FLAG-aGFP_{6M}-Halo and its complexes such as FLAG-aGFP_{6M}-Halo and GFP-POI could be induced. More importantly, such an L-AdPROM system could mediate an effective degradation (around 50%) of endogenous RAS via FLAG-Halo-aH/KRAS, indicating its potential for degrading any intracellular POI. Similarly, a Cas9 CRISPR-based TRAnscription Factor Targeting Chimeras (TRAFTACs) was recently developed from the haloPROTACs platform (Samarasinghe *et al.*, 2021). A chimeric oligo including transcription factor of interesting (TOI) binding double-stranded DNA (dsDNA) and Cas9 CRISPR-binding RNA (CR-RNA) was prepared first. As a linker, this chimeric oligonucleotide could connect TOI and dCas9-HaloTag7 fusion protein (dCas9HT7). With the attendance of haloPROTAC and VHL-E3 ligase, TRAFTACs could effectively trigger the ubiquitination and proteasomal degradation of oncogenic TOI, such as NF- κ B and brachyury. The brachyury targeting TRAFTACs was able to affect the formation of tails in zebrafish, showing a successful implementation of TRAFTACs for *in-vivo* application.

Other Applications of HaloTag

Notably, biomolecules on the cellular surface play crucial roles in numerous biochemical progress, ranging from immune response to cell differentiation. In addition to cell tracking or immunization via the fusion of HaloTag on the cell membrane, this site-specific binding domain is potentially accessible to a series of functional chemo/biomolecules. Several kinds of research were conducted to study cell development or functional analysis (Pulsipher *et al.*, 2018; Liu *et al.*, 2019). These assays incorporated a HaloTag protein into a cell membrane as surface anchors for further labeling. For example, Liu *et al.* (2019) labeled HeLa cells with a specific glycopolymer carrying acetylglucosamine and N-acetylmannosamine units, respectively (pMAG or pMAM) via membrane-merged HaloTag, which was able to persist for one week. After incubations with immune cells (e.g., macrophage or dendritic cells), it was observed that binding polymers could efficiently promote M1 marker expression (CD86 and iNOS), dendritic cell maturation and secretion of cytokines (TNF- α and IL-12p70), showing an enhanced anti-cancer effect. Similarly, Ohno *et al.* (2014) established a membrane-binding model via HaloTag fusion to investigate the development of stem cell pluripotent embryonic stem cells with attendance of heparan sulfate (HS) glycosaminoglycans (GAGs) (Pulsipher *et al.*, 2018).

Conclusion and Future Perspectives

Compared with other strategies (e.g., GFP and His-tag), the HaloTag platform offers several advantages for researches

requiring high accuracy and specificity. However, there are also several challenges related to the HaloTag system, of which researchers should be particularly attentive. (a) Although the modification will improve its solubility, the structure of HTL (hydrophobic) may still affect its circulation, especially for *in vivo* applications; in addition to innovative designs of HTLs, the pre-loading strategy (e.g., HaloTag-POI labeled with HTL *in vitro* before injection) may act as a potential solution; in contrast, long-term imaging could be also achieved via multiple administration of imaging HTL once high solubility was achieved. (b) Most HTLs used in studies are self-synthesized according to the purpose of their use. In other words, working knowledge and toolset for synthetic chemistry is a basic requirement for using the HaloTag system, which would limit its availability to some extent, such as for some biological labs; for this reason, we suspect that interdisciplinary cooperation may be a trend for future high-quality studies supported by HaloTag technique; (c) As the major feature, HaloTag technique could grant biomolecule with new chemical function via specific labeling and allow this a molecule act as temporal and spatial control. (d) Novel strategies like split HaloTag would promote efficiency and ensure accuracy for monitoring protein and protein interaction (Shao *et al.*, 2021). (e) Given that most studies of HaloTag technology depend on genetic engineering on targeting POI, further applications in clinical trials (which gene-editing could not be easily achieved) are directly hindered. However, the 1:1 ratio labeling capacity greatly ensures the reproduction of complex of HaloTag-fused protein and HTLs, such as PET tracer (i.e., HaloTag fused nanobody and radioactive isotope-labeled HTLs), well meeting the requirements of clinical application. Moreover, such PET tracers could be used for imaging and monitoring the diseases (e.g., tumor) or prognosis in real-time. (f) Besides, changeable HTLs may also offer the potential for the HaloTag-based tracers development for various applications. For instance, the HTLs-ICG conjugation to HaloTag-fused SCFV could be used for imaging guidance surgery. (g) Although the relatively larger size of HaloTag (33 kDa) may be one drawback compared with other more petite tags, like SNAP-tag (19.4 kDa), it may turn into an advantage when it is applied in the integration with SCFV (small-sized protein with short circulation), which may show a better enhancement in the circulation. In this case, extended monitoring could be successfully achieved. Therefore, we believe that the interdisciplinary cooperation and novel HTLs design would greatly promote the future development of HaloTag system, ensuring its efficiency in supporting high accuracy studies (e.g., PET-imaging tracer design).

Authors' Contribution: WYC planned and wrote this review with the help of ZKZ and WBC. MHY helped the writing. All authors contributed to the article and approved the submitted version.

Funding Statement: The authors are grateful for financial support from the University of Wisconsin—Madison and National Institutes of Health (P30CA014520).

Conflicts of Interest: Weibo Cai is a scientific advisor, stockholder, and grantee of Focus-X Therapeutics, Inc. All

other authors declare that they have no conflicts of interest to report regarding the present study.

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