HaloTag™ Interchangeable Labeling Technology for Cell Imaging, Protein Capture and Immobilization

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Abstract

The HaloTag™ Interchangeable Labeling Technology is designed to provide new options for rapid, site-specific labeling of proteins in living cells and in vitro. The technology is based on the efficient formation of a covalent bond between the HaloTag™ Protein and synthetic ligands. The covalent bond forms rapidly under physiological conditions, is highly specific and essentially irreversible, yielding a stable complex even under denaturing conditions. The HaloTag™ Ligand can carry a variety of functionalities, including fluorescent labels, affinity handles and attachments to a solid phase. The flexibility to create labeled HaloTag™ fusion proteins with a wide range of optical properties and functions will allow researchers to image and localize labeled HaloTag™ Protein fusions in live- or fixed-cell populations as well as isolate and analyze HaloTag™ Protein fusions and protein complexes.

The interchangeability of the HaloTag™ Ligands facilitates imaging at different wavelengths or incorporating novel functionalities without changing the underlying genetic construct.

Introduction

The ability to specifically label proteins is key to revealing the dynamics and functions of proteins in living cells (1). However, many conventional methods used to generate fluorescently tagged proteins and image them in their native environment are time consuming and difficult, requiring expertise in protein chemistry, the successful microinjection of labeled products into cells, and specialized techniques and instrumentation (2). On the other hand, de novo synthesis of proteins obtained through cloning and transfection of cells is more likely to result in native patterns of protein localization. The development of new methods for labeling proteins by genetic fusion is expanding our understanding of cellular function. Here we describe the new HaloTag™ Interchangeable Labeling Technology, a flexible system that enables efficient labeling of fusion proteins in living cells as well as in vitro.

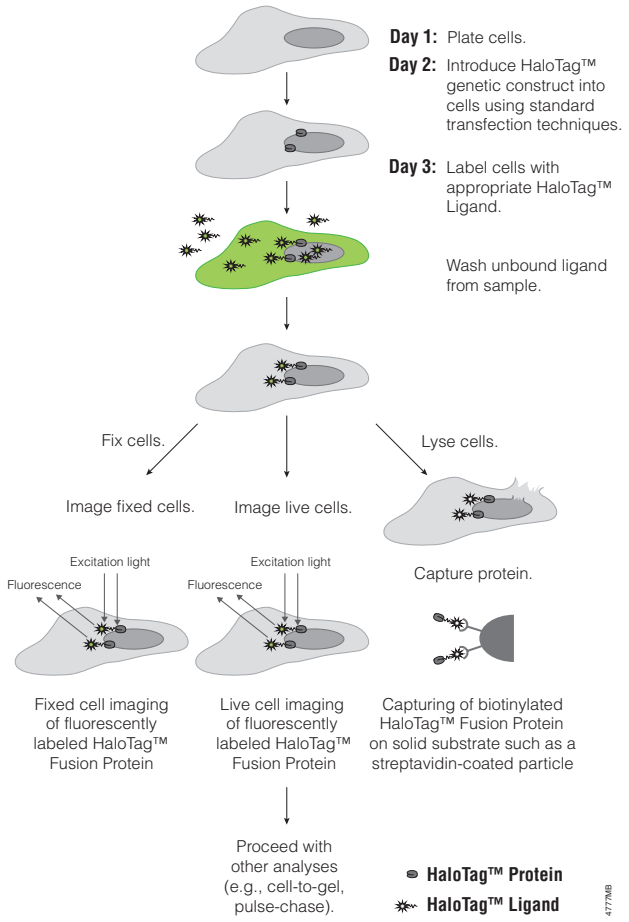

Figure 1. Overview of the HaloTag™ Interchangeable Labeling Technology applications.

Overview of the HaloTag™ Interchangeable Labeling Technology

Figure 1 provides an overview of the HaloTag™ Labeling strategy. First, the HaloTag™ pHT2 Vector[(a,b,c)] encoding the HaloTag™ Protein or protein fusion is introduced into cells. A variety of standard methods may be used for either transient transfection or generation of stable cell lines expressing the HaloTag™ Protein. The cells are typically incubated with 5–25µM of an appropriate HaloTag™ Ligand[(c,d)] for 5–60 minutes. The HaloTag™ Ligands readily cross the membrane where they covalently bind to the HaloTag™ Protein. Unbound ligand is washed out, and the investigator proceeds with the desired application: fluorescence imaging (live- or

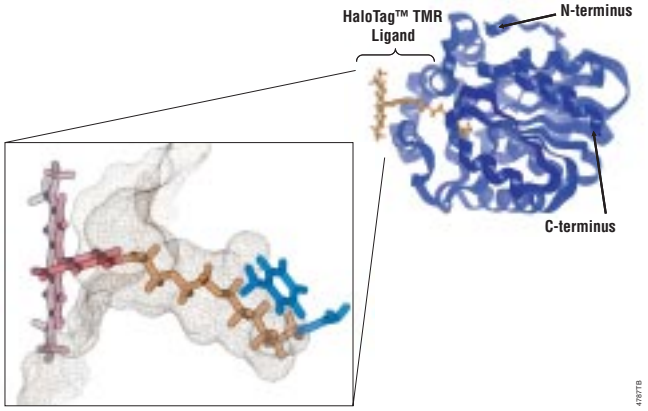

Figure 2. Molecular model of the HaloTag™ Protein with a covalently bound HaloTag™ TMR Ligand. Overview of the protein structure (top right) with close-up of the ligand tunnel outlined by a mesh Connolly surface. The HaloTag™ TMR Ligand (functional group in red, reactive linker in orange) is shown covalently bound to the aspartate nucleophile (shown in blue). Replacement of the catalytic base (histidine) with a phenylalanine residue (also shown in blue) renders the HaloTag™ Protein catalytically inactive by impairing its ability to hydrolyze the ester intermediate leading to the formation of a stable covalent bond.

fixed-cell), cell lysis and protein capture, or gel analysis. The interchangeability of fluorescent ligands facilitates imaging at different wavelengths or incorporating novel functionalities without changing the underlying genetic construct. Detailed protocols are provided in the *HaloTag™ Interchangeable Labeling Technology Technical Manual*, #TM260.

Components of the HaloTag™ Interchangeable Labeling Technology

The HaloTag™ Protein is a catalytically inactive, genetically modified derivative of a hydrolase protein that efficiently forms a covalent bond with the HaloTag™ Ligands (Figure 2). This 33kDa monomeric protein can be used to generate N- or C-terminal fusions that can be efficiently expressed in a variety of cell types. Since the HaloTag™ Protein is of prokaryotic origin, endogenous activities are absent from mammalian cells. The HaloTag™ pHT2 Vector contains: a CMV enhancer/promoter for strong, constitutive expression in many cell types, a chimeric intron to minimize the use of cryptic 5′-donor splice sites, a T7 promoter for use with in vitro transcription and/or translation systems, and an SV40 late polyadenylation signal.

The HaloTag™ Ligands are small chemical tags that are capable of covalently labeling the HaloTag™ Protein. These ligands contain two crucial components, 1) a common HaloTag™ Reactive Linker that initiates formation of a covalent bond with the HaloTag™ Protein, and 2) a functional reporter such as the fluorescent dyes TMR and diAcFAM or affinity handles such biotin (Figure 3). The rate of HaloTag™ Ligand binding to the HaloTag™ Protein is remarkably fast. Fluorescence polarization analysis using a purified GST-HaloTag™

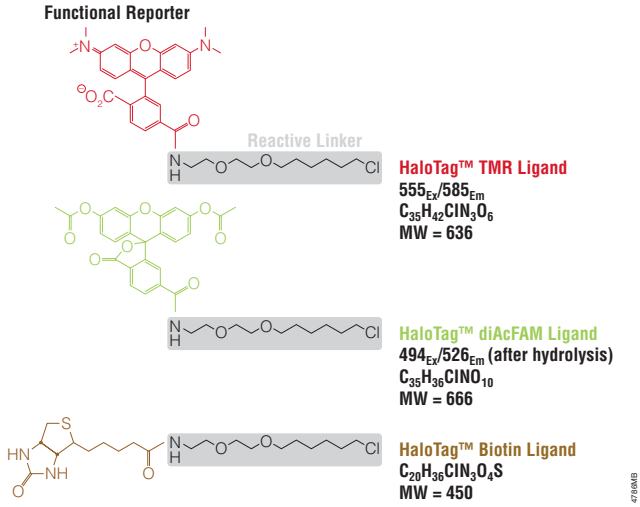

Figure 3. Structure of the HaloTag™ Ligands. Abbreviations: TMR, tetramethyl-rhodamine; diAcFAM, diacetyl-fluorescein.

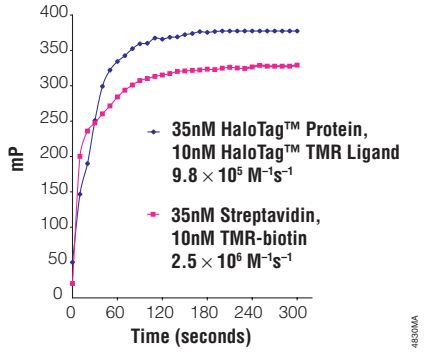

Figure 4. Fluorescence polarization (FP) analysis comparing HaloTag™ Ligand and streptavidin-biotin on-rates. Measurements were performed on a Beacon 2000 instrument, and the data were used to calculate second-order rate constants (3). Qureshi *et al.* (4) have reported streptavidin-biotin on-rates of 5.0×10^6 M^{-1} sec^{-1}.

Protein fusion shows that the HaloTag™ TMR Ligand binds in vitro very quickly. The binding kinetics are similar to the on-rate measured for TMR-biotin binding to streptavidin (Figure 4). This rate is similar to the value reported for the biotin-streptavidin interaction (4). The HaloTag™ TMR, diAcFAM and Biotin Ligands have shown no detectable cellular toxicity or morphological side effects under the labeling conditions described in Technical Manual #TM260.

Imaging Live and Fixed Mammalian Cells

As shown in Figure 5, cells expressing the HaloTag™ Protein labeled with the HaloTag™ TMR or HaloTag™ diAcFAM Ligand are brightly fluorescent. In contrast, control cells lacking the HaloTag™ Protein show no detectable fluorescence under identical imaging conditions. These results demonstrate the high specificity of cell labeling and the efficient use of both ligands for imaging live cells. The excellent signal-to-noise ratio of

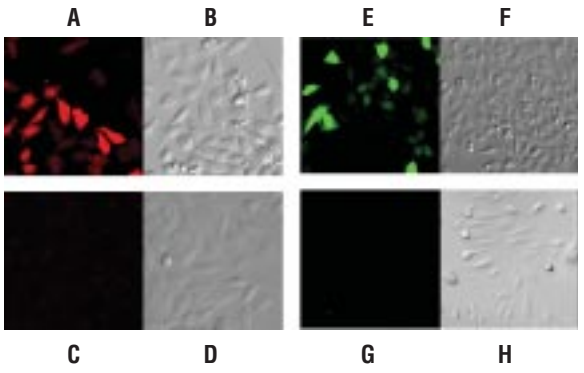

Figure 5. Bright fluorescence images of live cells expressing HaloTag™ Protein labeled with HaloTag™ TMR Ligand or HaloTag™ diAcFAM Ligand. HeLa cells transiently transfected with HaloTag™ pHT2 Vector (**Panels A, B, E and F**) or control cells (**Panels C, D, G and H**) were labeled with 5µM HaloTag™ TMR Ligand (**Panels A–D**) or 10µM HaloTag™ diAcFAM Ligand (**Panels E–H**) for 15 minutes at 37°C. Images were generated on an Olympus FV500 confocal microscope using appropriate TMR and FAM filter sets or transmitted light.

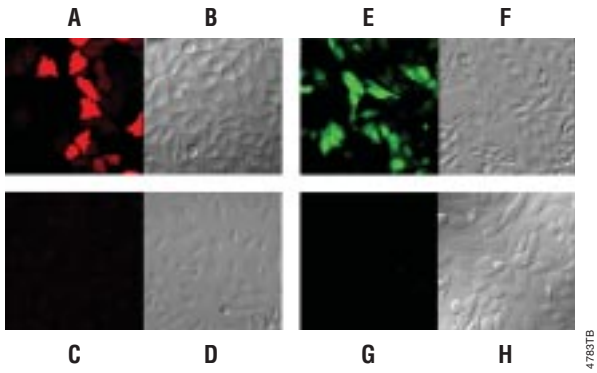

Figure 6. Bright fluorescence images of fixed cells expressing HaloTag™ Protein labeled with HaloTag™ TMR Ligand or HaloTag™ diAcFAM Ligand. HeLa cells transiently transfected with HaloTag™ pHT2 Vector (**Panels A, B, E and F**) or control cells (**Panels C, D, G and H**) were labeled with 5µM HaloTag™ TMR Ligand (**Panels A–D**) or 10µM HaloTag™ diAcFAM Ligand (**Panels E–H**) according to the protocol outlined in Figure 1. Cells were fixed with 3.7% paraformaldehyde, and images were generated on the Olympus FV500 confocal microscope using appropriate fliter sets for TMR and FAM or transmitted light (**Panels B, D, F and H**).

the fluorescence signal should allow detection of those proteins expressed at relatively low levels.

The stability of the covalent bond between the HaloTag™ Protein and the HaloTag™ Ligands easily allows fixed-cell imaging. HeLa cells expressing the HaloTag™ Protein were labeled with the HaloTag™ TMR or HaloTag™ diAcFAM Ligand. Transfected cells remain brightly fluorescent upon fixation (Figure 6). However, no fluorescence is detected in control, ligand-treated cells. The resistance of the fluorescent signal to cell fixatives should make it possible to multiplex the HaloTag™ Technology with different immuno-cytochemical and immunohistochemical techniques. In addition, the ability to image fixed cells is also important for applications where large numbers of cells are analyzed by automated screening methods such as those involving High Content Analysis.

Gel Analysis of HaloTag™ Fusion Proteins Labeled in Live Cells

The HaloTag™ Protein-Ligand interaction is very stable. Fluorescently labeled HaloTag™ Protein can be boiled with SDS sample buffer and resolved by SDS-PAGE without detectable loss of the fluorescent signal. This attribute is particularly useful when the labeling of HaloTag™ fusion proteins in living cells is combined with SDS-PAGE and fluorescence image analysis.

CHO-K1 cells transiently expressing HaloTag™ Protein were incubated with the HaloTag™ TMR Ligand in a time course experiment. Cell lysates analyzed by SDS-PAGE revealed a single fluorescent band with a molecular weight corresponding to the HaloTag™ Protein (Figure 7). While labeling of all available HaloTag™ binding sites in living cells was achieved in as little as 5 minutes, this is still considerably slower than the rate observed in vitro (Figure 4). This may indicate that the transport of ligands through the cell membrane is rate-limiting. Analyzing labeled HaloTag™ Protein fusions directly from cells (cell-to-gel) can be combined with other protein analysis techniques such as Western blot analysis. Our preliminary data also indicate that this approach can be used successfully to study post-translational modification of HaloTag™ Protein-based fusions (e.g., proteolytic cleavage, data not shown).

Capture of HaloTag™ Protein Fusions

HaloTag™ Labeling Technology can also be used to capture HaloTag™ Protein fusions expressed in mammalian cells using a ligand containing an affinity handle. CHO-K1 cells transiently transfected with a construct encoding a *Renilla* luciferase-HaloTag™-FLAG protein fusion were incubated in the presence or absence of the HaloTag™ Biotin Ligand according to the protocol described in Technical Manual #TM260. Cell lysates were incubated with Streptavidin MagneSphere® Paramagnetic Particles (Cat.# Z5481) to capture the biotin-labeled fusion protein. Proteins were resolved by SDS-PAGE and analyzed by Western blot using an anti-FLAG antibody. As expected, both ligand-treated and control cells express *Renilla* luciferase-HaloTag™-FLAG protein (Figure 8, Panel A, lanes 1 and 4). However, only beads incubated with the cytosol of ligand-treated cells are able to capture *Renilla* luciferase-HaloTag™-FLAG protein (Figure 8, Panel A, lanes 3 and 6). Importantly, the fusion protein captured on the beads retains *Renilla* luciferase activity (Figure 8, Panel B).

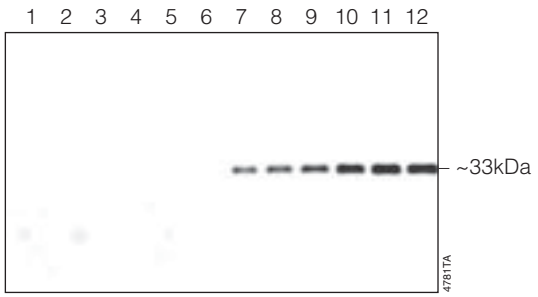

Figure 7. Fast, efficient and highly specific labeling of the HaloTag™ Protein expressed in mammalian cells. CHO-K1 control cells (lanes 1–6) or cells transiently transfected with HaloTag™ pHT2 Vector (lanes 7–12) were labeled with 5µM HaloTag™ TMR Ligand for different periods of time at 37°C (0.5, 1, 2, 5, 15 and 30 minutes). Following labeling the medium was removed, and the cells were quickly rinsed with PBS prior to lysis with SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE and analyzed on a Hitachi FMBIO® fluorescence scanner.

Conclusion

The HaloTag™ Interchangeable Labeling Technology is designed to create labeled proteins in living cells or in vitro with a potentially wide range of optical properties and functionalities. This new technology can be used to image live or fixed mammalian cells, allowing users to monitor the intracellular translocation of HaloTag™ Protein fusions, targeted subcellular structures or protein turnover. The technology can also be used to examine various post-translational events using SDS-PAGE (cell-to-gel) analysis or to capture HaloTag™ Protein fusions and protein complexes directly from living cells or in vitro transcription/translation reactions onto solid supports. The system can readily be applied to different cell lines and organisms thereby providing a universal, yet integrated experimental approach for the study of proteins and protein function.

References

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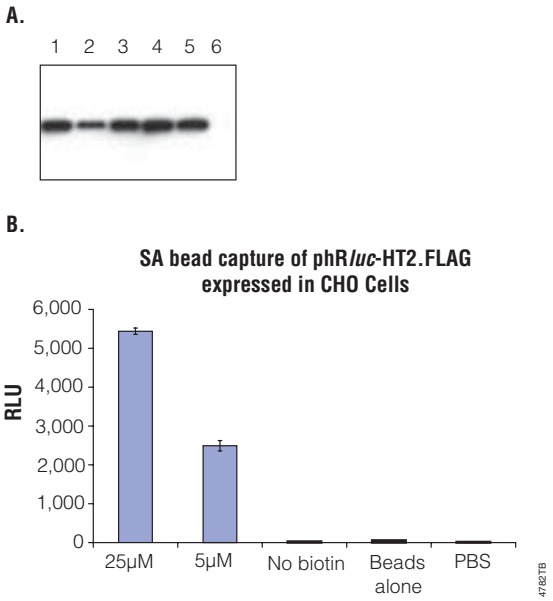

Figure 8. Efficient capture of a HaloTag™ Protein-based fusion on paramagnetic streptavidin (SA)-coated particles. CHO-K1 cells transiently transfected with the HaloTag™ pHT2 Vector coding for a *Renilla* luciferase-HaloTag™-FLAG protein fusion were incubated in the presence (5µM or 25µM; 15 minutes, 37°C) or the absence of HaloTag™ Biotin Ligand. Cells were lysed, and biotin-labeled protein was captured on SA-coated paramagnetic particles (Cat.# Z5481). **Panel A.** Western blot analysis with anti-FLAG antibody M2 (Sigma). Lane 1, cell lysate (+ ligand); lane 2, unbound proteins (+ ligand); lane 3, bound proteins (+ ligand); lane 4, cell lysate (– ligand); lane 5, unbound proteins (– ligand); lane 6, bound proteins (– ligand). **Panel B.** Activity of captured luciferase was measured on an Orion Microplate Luminometer (Berthold Detection Systems) using the *Renilla* Luciferase Assay Substrate (Part# E289B).

Protocol

◆ *HaloTag™ Interchangeable Labeling Technology Technical Manual* #TM260, Promega Corporation.
www.promega.com/tbs/tm260/tm260.html

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Ordering Information

Product	Size	Cat.#
HaloTag™ pHT2 Vector	20µg	G8241
HaloTag™ TMR Ligand	30µl	G8251
HaloTag™ diAcFAM Ligand	30µl	G8271
HaloTag™ Biotin Ligand	30µl	G8281

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