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The HaloTag™

A Novel Technology for Cell Imaging and Protein Analysis

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Summary

The ability to specifically label proteins with a wide range of optical properties and functionalities can help reveal information about protein functions and dynamics in living cells. Here, we describe a technology for covalent tethering of organic probes directly to a specially designed reporting protein expressed in live cells. The reporting protein can be used in a manner similar to green fluorescent protein, except that the fluorophore might be interchanged among a variety of standard dyes. This allows living cells to be imaged at different wavelengths without requiring changes to the underlying genetic constructs, and the colors can be rapidly switched to allow temporal analysis of protein fate. The stability of the bond permits imaging of live cells during long time periods, imaging of fixed cells, and multiplexing with different cell/protein analysis techniques. The dyes can also be exchanged with other functional molecules, such as biotin to serve as an affinity handle, or even solid supports for direct covalent immobilization. The technology complements existing methods and provides new options for cell imaging and protein analysis.

Key Words: Cell-based; covalent; fluorescence; functional groups; imaging; interchangeable; microscopy; protein capture; protein labeling; reporters; site-specific; synthetic ligands.

1. Introduction

Specific labeling of proteins in living cells, combined with noninvasive detection techniques, can be a useful strategy for revealing functional and dynamic attributes within complex intracellular environments. However, achieving such labeling can be technically challenging. Here we describe the HaloTag™ Interchangeable Labeling Technology for covalent site-specific tethering of synthetic ligands to a fusion reporter protein in living cells. The reporter protein is an engineered, catalytically inactive, derivative of a bacterial hydrolase (**Fig. 1**, *see also* **Appendix**). The ligands are small synthetic molecules carrying various functionalities, such as fluorescent labels or affinity handles (**Fig. 2**). Ligands attached onto a solid surface might also be used for protein immobilization. The open design of the technology ensures interchangeability for a broad range of ligand structures, thereby facilitating a variety of functional studies without requiring changes to the underlying genetic construct. For example, fluorescent imaging might be done at a choice of wavelengths as specified by changing experimental requirements. The covalent bond to the reporter protein forms rapidly under general physiological conditions, is highly specific and essentially irreversible, yielding a complex that is stable even under stringent conditions. The

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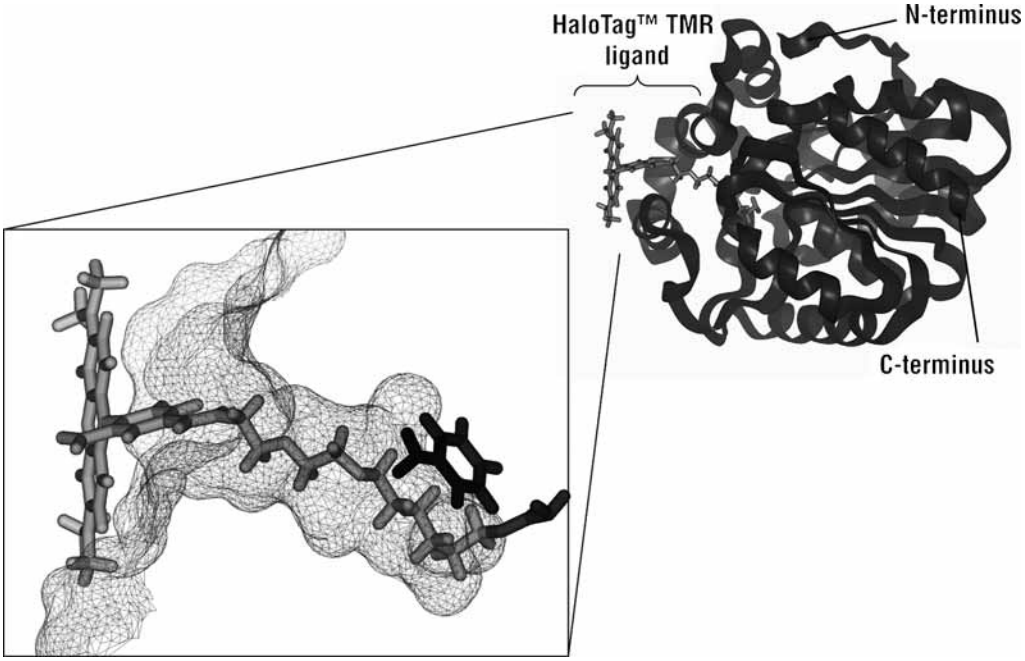

Fig. 1. Molecular model of the HaloTag protein with a covalently bound HaloTag™ TMR ligand.

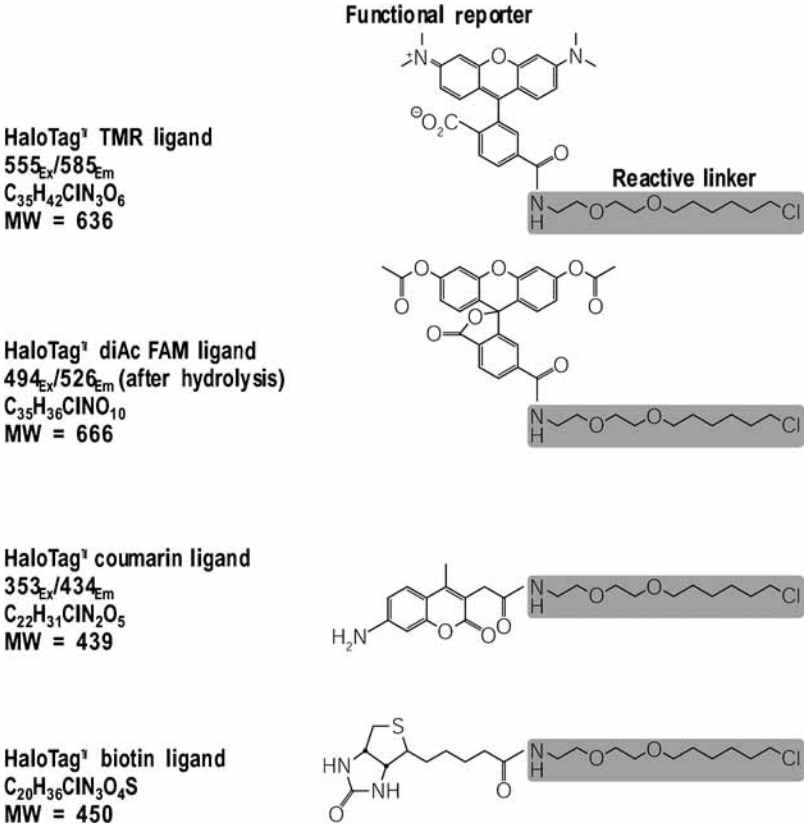

Fig. 2. Structure of the HaloTag™ Ligands. TMR, tetramethyl rhodamine; diAcFAM, diacetyl fluorescein. These HaloTag ligands readily cross the cell membrane and can be used for cell imaging or protein capture.

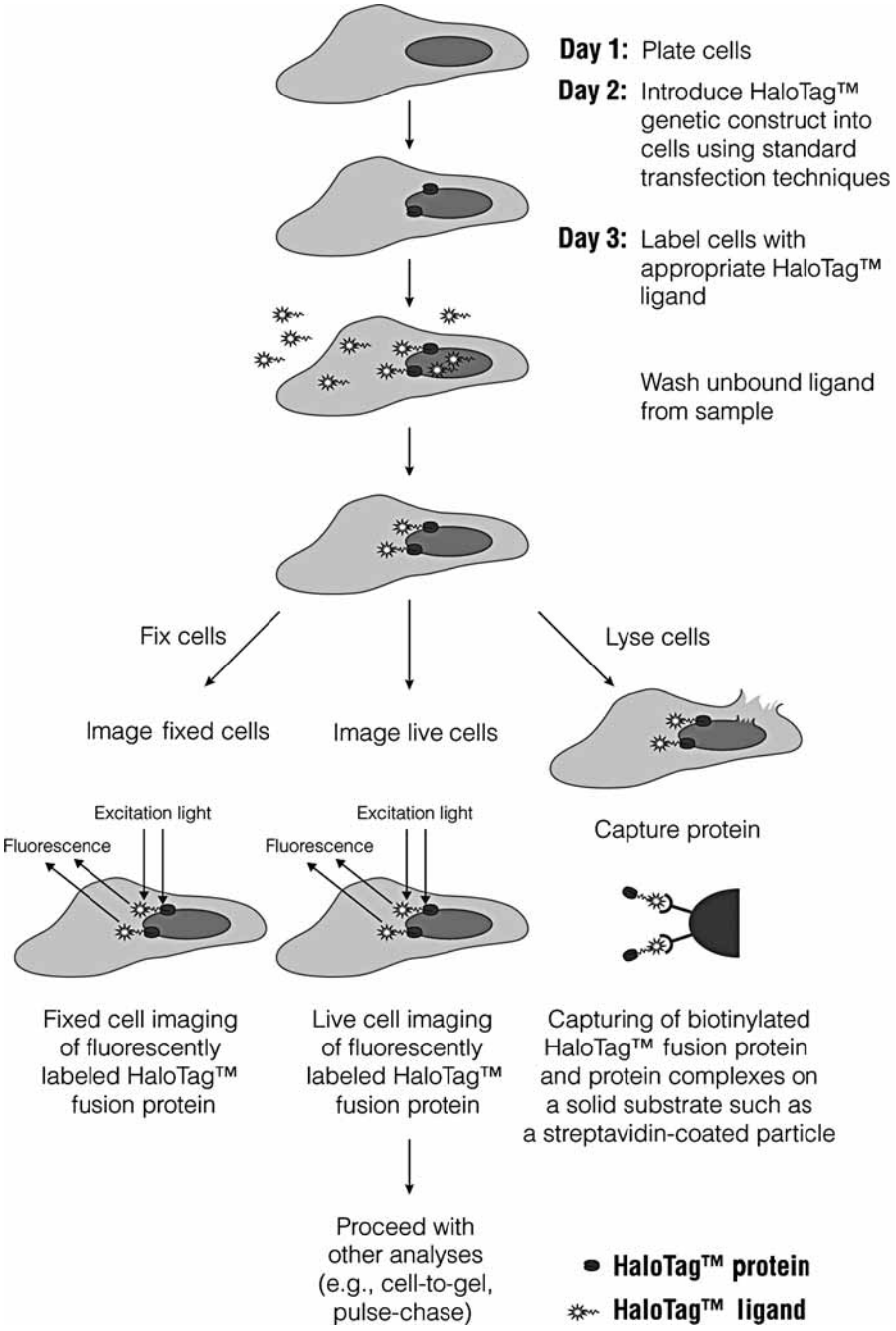

Fig. 3. Overview of cell-based applications for the HaloTag™ technology.

stability of the bond permits imaging of live cells during long time periods, imaging of fixed cells, and multiplexing with different cell/protein analytical techniques ***(1)***. The general process for cell-based application includes: (1) making a vector encoding a fusion of the HaloTag protein to a protein, protein-domain, or peptide sequence of interest; (2) expressing the fusion chimera in cells; (3) labeling the cells with the HaloTag ligand; and (4) imaging the sample, either as live or fixed cells (**Fig. 3**).

2. Materials

1. HaloTag pHT2 Vector (Promega Madison, WI).
2. HaloTag Ligands (Promega).
3. HeLa cells (ATCC, cat. no. CCL-2).
4. CHO-K1 cells (ATCC, cat. no. CCL-61).
5. Fetal bovine serum.
6. Serum-free cell culture medium.
7. 8-well Lab-Teck^{R}II chamber cover glass (Nalgen, Nunc).
8. Transfection reagents: Lipofectamine 2000 (Invitrogen) and LT1 transfection reagent (Mirus, Madison, WI).
9. Endotoxin-free (transfection grade) plasmid DNA.
10. PBS (37°C).
11. Wide-field or confocal fluorescent microscope.
12. 37°C cell culture incubator.
13. 3.7% paraformaldehyde containing 0.5 m*M* sucrose.
14. Triton® X-100.
15. 0.1% sodium azide/PBS solution.
16. SDS-polyacrylamide gels and associated buffers and stains, as well as electroblot transfer buffers (BioWhittaker Molecular Applications; Rockland, ME).
17. Protein molecular weight standards (Invitrogen).
18. Anti-p65 antibody (BD Biosciences).
19. Anti-IκB antibody (BD Biosciences).
20. Antiβ-III Tubulin Antibody (Promega).
21. Antimouse IgG, conjugated with AlexaFluor™-488 (Invitrogen).
22. HRP-conjugated goat anti-mouse IgG (Promega).
23. Enhanced chemiluminescence system (Pharmacia-Amersham).
24. Sample buffer: 1% SDS, 10% glycerol, and 1 m*M* β-mercaptoethanol, pH 6.8.
25. Transfer buffer: 25 m*M* Tris base/188 m*M* glycine pH 8.3, 20% (v/v) methanol.
26. TBST buffer: 10 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.6, 0.05% Tween-20.
27. Blocking solution: 3% dry milk or 1% BSA in TBST buffer.

3. Methods

The methods described next outline: (1) the description and design of expression plasmids; (2) culture, transfection, labeling with the HaloTag Ligands, fixing, and immunocytochemical analysis of mammalian cells; (3) imaging of live and fixed cell; (4) capture and characterization of protein.

3.1. Expression Plasmids

This section describes the HaloTag pHT2 Expression Vector, and a design of the expression plasmids for a HaloTag·$(NLS)_3$ (the HaloTag Protein targeted to nucleus) and p65-HaloTag fusion protein, which has cytosolic localization (i.e., excluded from nucleus) in nonstimulated cells.

3.1.1. HaloTag pHT2 Expression Vector

The HaloTag pHT2 Expression Vector (**Fig. 4A,B**) contains the open reading frame of the modified hydrolase gene cloned into a mammalian expression vector with the following features:

- Kozak sequence for translation initiation was added to the beginning of the gene.
- The following restriction sites were added for convenience:
    - o *Bam*HI and *Nae*I restriction sites were added within the HaloTag gene coding region to allow convenient creation of protein fusions. The *Bam*HI site is located immediately after the ATG start codon and the *Nae*I site is located just before the stop codon.
    - o The other restriction sites were added just outside of the coding region. Near the N-terminus are *Nhe*I, *Pvu*II, *Eco*RV, and a nonunique *Nco*I; near the C-terminus are *Pac*l and *Not*I. The *Eco*RV site cuts the pHT2 Vector in frame with the HaloTag coding region.

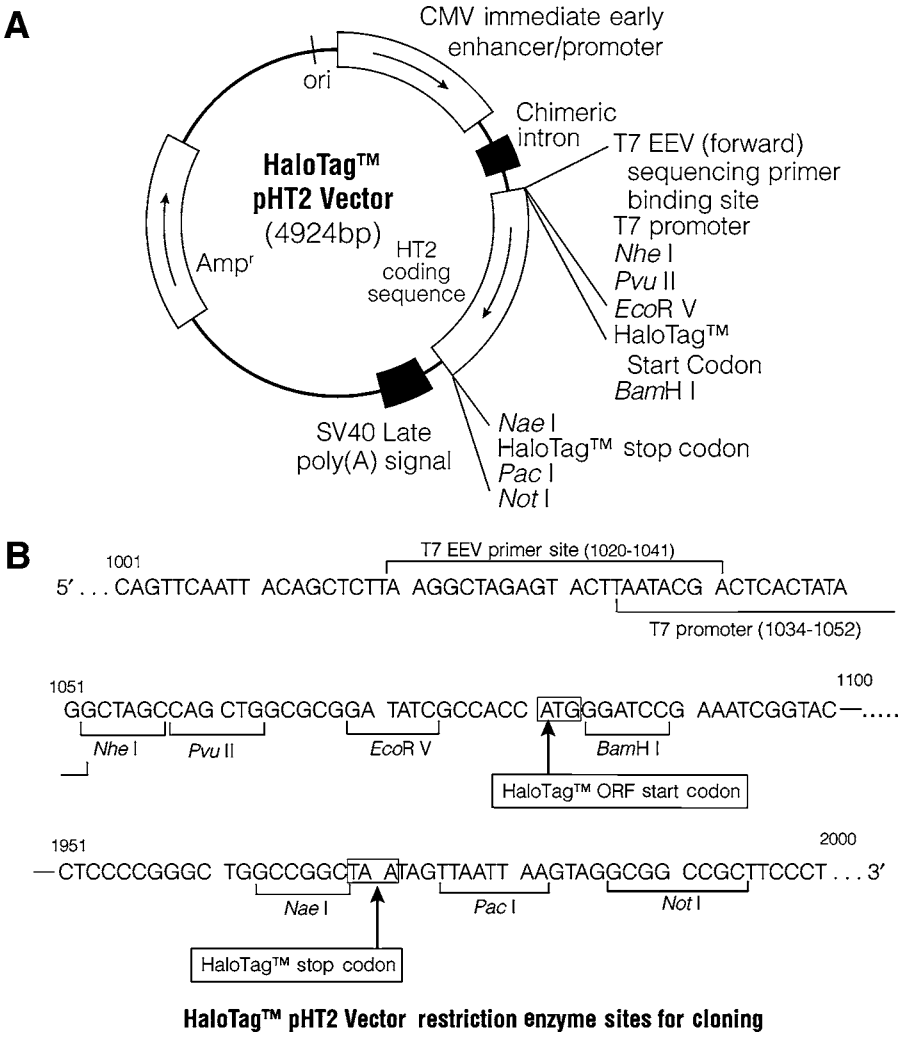

Fig. 4. (**A**) HaloTag™ pHT2 vector circle map and sequence reference points, and (**B**) potential cloning sites and primer hybridization near the start and stop codons of the coding region.

When designing fusions to the HaloTag Protein, we recommend inserting a polypeptide linker between the fusion partners to reduce the potential for structural hindrance. The size and the sequence of the polypeptide linker should be determined empirically, although a 17-amino acid linker comprising a repeated series of glycines and serines has served as a good starting point in our experiments.

3.1.2. Design of the HaloTag·(NLS)$_3$ and p65- HaloTag Expression Vectors

The HaloTag Protein can be fused to a protein of interest, such as one that will direct the fusion protein to a specific subcellular location. To demonstrate this capability we generated expression vectors encoding the HaloTag·(NLS)$_3$ and the p65-HaloTag·FLAG fusion proteins (**Fig. 5**). An (NLS)$_3$ is three repeats of a nuclear localization sequence from simian virus large T-antigen (*2*). The p65 protein (also known as RelA and NF-κB3) is a member of the eukaryotic nuclear factor-κB (NF-κB)/Rel transcription factor protein family. The NF-κB proteins contain a nuclear-localization sequence (NLS) that is rendered inactive in nonstimulated cells through

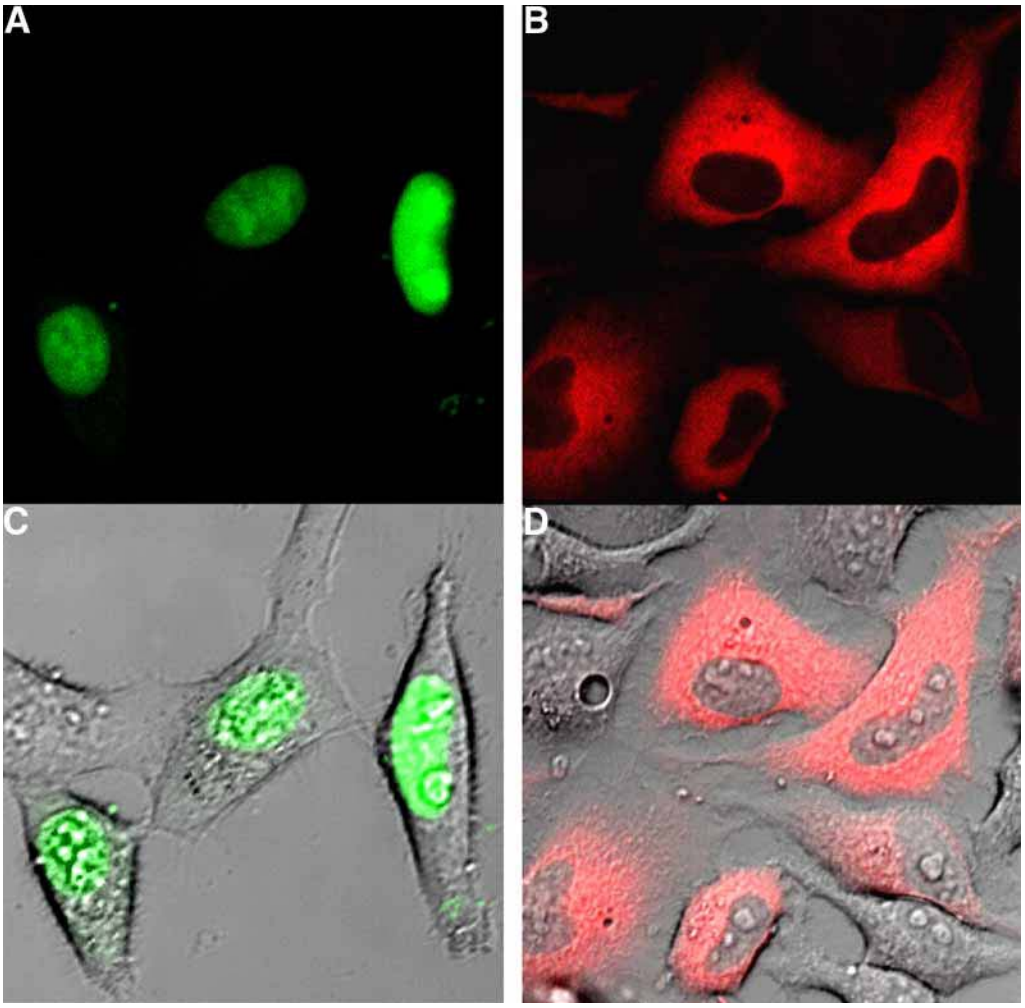

Fig. 5. Nuclear localization of the HaloTag™ $(NLS)_3$ protein and cytosolic localization of the p65-HaloTag·FLAG fusion protein labeled with the HaloTag Ligands. HeLa cells were transiently transfected with the vector encoding the HaloTag·$(NLS)_3$ (**A,C**) or the p65-HaloTag·FLAG (**B,D**) fusion protein were labeled with 5 μ*M* HaloTag diAcFAM Ligand (**A,C**) or HaloTag TMR Ligand (**B,D**) for 15 min at 37° C. Images were generated on an Olympus FV500 confocal microscope using appropriate filter sets for TMR and FAM or transmitted light. (**C**) Overlaid FAM fluorescence and transmitted light. (**D**) Overlaid TMR fluorescence and transmitted light.

the binding of specific NF-κB inhibitors, known as the IκB proteins. Binding of IκB masks the NLS, which leads to retention of NF-κB proteins (including the p65-p50 heterodimer) in the cytoplasm of the cells *(3–5)*.

The expression vector encoding the HaloTag·$(NLS)_3$ fusion protein was generated by subcloning the coding sequence of $(NLS)_3$ (5´-GA TCCAAAAAAG AAGAGAAAGG TAGATC-CAAA AAAGAAGAGA AAGGTAGATC CAAAAAAGAA GAGAAAGGTA-3´) in frame at the C-terminus of the HaloTag Protein.

Plasmid encoding p65 was generously provided by Dr. Johannes A. Schmid *(6)*. The expression vector encoding the p65-HaloTag·FLAG fusion protein was generated by subcloning the coding sequence for the sequence of p65 in frame at the N-terminus of the HaloTag Protein. To reduce

potential three-dimensional structural hindrance effects of the fusion partners, a 15-amino acid flexible polypeptide linker was inserted between p65 and the HaloTag Protein. The sequence of the linker is: LDPLVTRGTSRVDAA (5´-TTG GAT CCA CTA GCT ACG CGT GGT ACC TCT AGA GTC GAC GCC GCC-3´). The resulting clones were confirmed by DNA sequencing.

3.2. Culture, Transfection, and Labeling With the HaloTag Ligands

1. Maintaining healthy cell culture is essential for all mammalian cell-based applications. Culturing conditions for many cell lines (e.g., CHO-K1 or HeLa) are available from the cell supplier (e.g., ATCC). For additional information, consult **refs.** *7* and *8*.
2. A variety of methods might be used for transient transfection for example lipofection *(9,10)*, calcium phosphate *(11,12)*, electroporation *(13,14)*, or viral *(15,16)*. Stable cell lines expressing HaloTag Protein or HaloTag Protein-based fusions also can be generated (*see* **Notes 1** and **2**).
3. All procedures described in this chapter were performed at the standard cell growing conditions, i.e., at 37°C, in atmosphere of 5% CO_2. HeLa cells or CHO-K1 cells were maintained in DMEM/F12 media supplemented with 10% fetal bovine serum (Invitrogen) according to ATCC recommendations.
4. To study transient expression of different proteins, cells were plated on 8-well Lab-Teck^{R}II chamber cover glass (Nalgen, Nunc) at seeded density of $7.5–10 \times 10^3$ cells/cm^2 ($9–12 \times 10^3$ cells/cm^2) in 400 µL growth medium, and allowed to grow to approx 85% confluency (approx 24–48 h).
5. Cells were transfected using Lipofectamine 2000 or LT1 transfection reagents according manufacturer's protocols.
6. The HaloTag TMR, HaloTag diAcFAM, and HaloTag Biotin Ligands readily cross the cell membrane, allowing labeling and detection of the HaloTag Protein in living mammalian cells (*see* **Note 3**).
7. The HaloTag Ligands are commercially available as 5 or 10 m*M* stock solutions in DMSO. Ligands are dispensed into aliquots and stored at –20°C, desiccated and protected from light.
8. The growth medium on cultured cells was replaced with growth medium containing the appropriate ligand (200 µL/well). The recommended working concentration in medium are: 1–5 µ*M* for HaloTag TMR Ligand; 1–10 µ*M* for HaloTag diAcFAM Ligand, and 5–10 µ*M* for HaloTag Biotin Ligand and HaloTag Coumarin Ligand.
9. After 15 min, cells were rinsed twice with PBS (pH 7.4), incubated in fresh media for 30 min, then media was replaced with fresh media or PBS, and cells were used for imaging or protein capture.

3.3. Fixing Mammalian Cells and Immunocytochemistry

The stability of the covalent bond between the HaloTag Protein and the HaloTag Ligands allows imaging the HaloTag fusion proteins in fixed cells (**Fig. 6**). In addition, the resistance of the fluorescent signal to cell fixatives also allows multiplexing the HaloTag Technology with different immunocytochemical and, potentially, immunohistochemical techniques (**Fig. 7**). In this section we describe fixing and immunocytochemical analysis of cells expressing HaloTag Proteins.

1. HeLa cells transiently transfected with the plasmid encoding the HaloTag fusion protein were labeled with the HaloTag TMR, the HaloTag diAcFAM, or HaloTag Coumarin Ligand as described earlier.
2. Cells were rinsed with PBS (37°C, 400 µL/well of 8-well Lab-Teck^{R}II chamber), PBS was replaced with 400 µL freshly prepared 3.7% paraformaldehyde containing 0.5 m*M* sucrose, and cells were incubated for 10 min at RT in the dark.
3. The fixative was replaced with PBS containing 0.1% Triton X-100, and cells were incubated for 30 min at RT in the dark.
4. The Triton X-100 solution was replaced with 400 µL PBS, 15 min, RT.
5. The cells were incubated in a blocking solution (1% BSA in TBST buffer: 10 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.6, containing 0.05% Tween-20) for 30 min at room temperature.
6. Cells were washed with TBST buffer (1.0 mL/cm^2, 15 min, RT) and incubated with mouse anti-βIII tubulin antibody at 1 µg/mL in TBST buffer for 45 min at RT.
7. Cells were washed with TBST buffer (1.0 mL/cm^2, three times, 15 min each, RT).
8. Primary antibody was visualized by incubation of the cells with Alexa Fluor™-488 conjugated goat-antimouse IgG (dilution 1:1000, 30 min, RT) and then the washing procedure was repeated. The cells were imaged immediately or were stored in a 0.1% sodium azide/PBS solution protected from light.

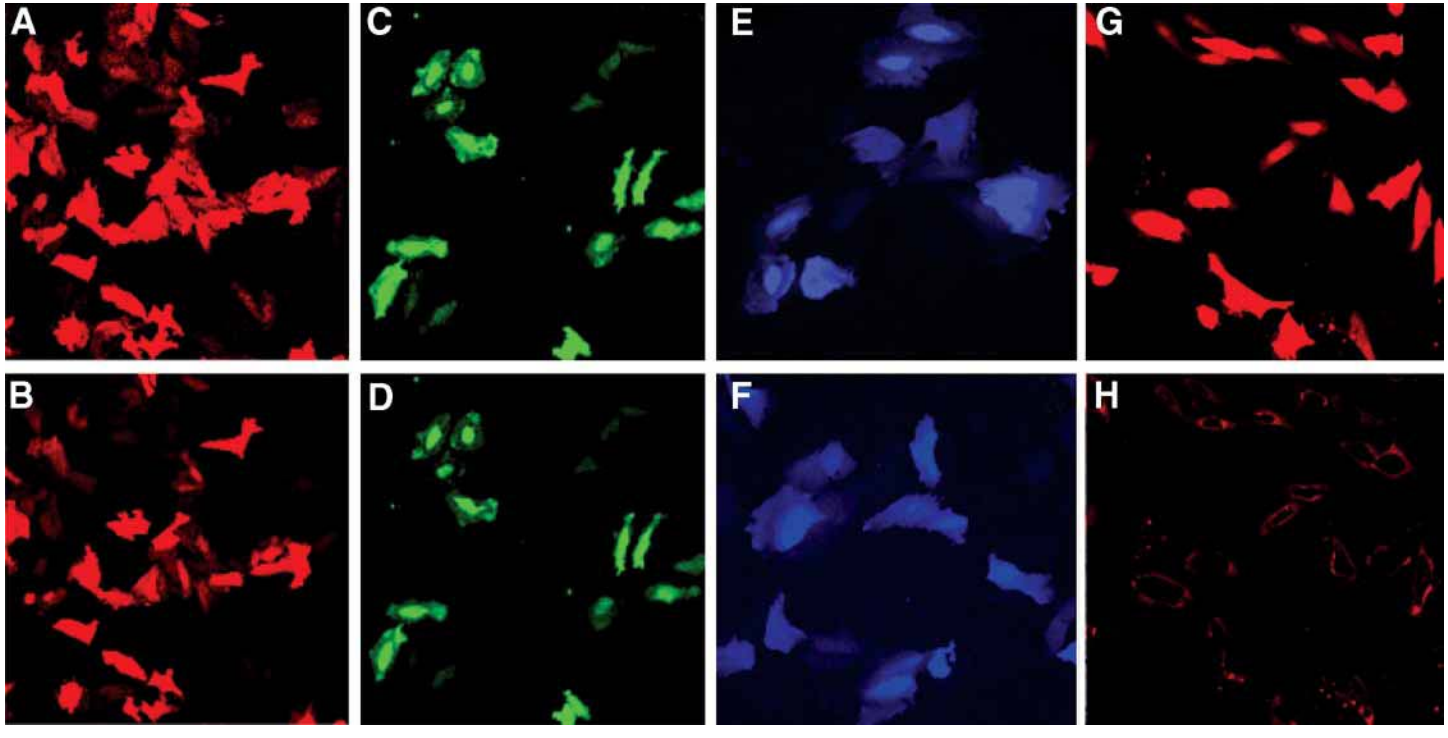

Fig. 6. The HaloTag™ ligand withstands fixation. HeLa cells were transiently transfected with the HaloTag pHT2 Vector **(A–F)** or a DsRed2 **(G,H)**. Twenty-four hours later, cells expressing the HaloTag Protein were labeled with 5 μ*M* HaloTag TMR ligand (**A,B**); 10 μ*M* HaloTag diAcFAM ligand (**C,D**); or 25 μ*M* HaloTag Coumarin ligand (**E,F**), for 15 min at 37° C/5% CO_2. Cells were imaged, fixed with 3.7% paraformaldehyde, and imaged again using identical microscope settings. Images in **A–D**, **G,H** were generated on an Olympus FV500 confocal microscope using appropriate filter sets for TMR or FAM, and show identical fields before and after fixation. Images in **E,F** were captured with Hamamatsu CCD camera with appropriate filter set for coumarin, and represent different fields of view within the same culture well.

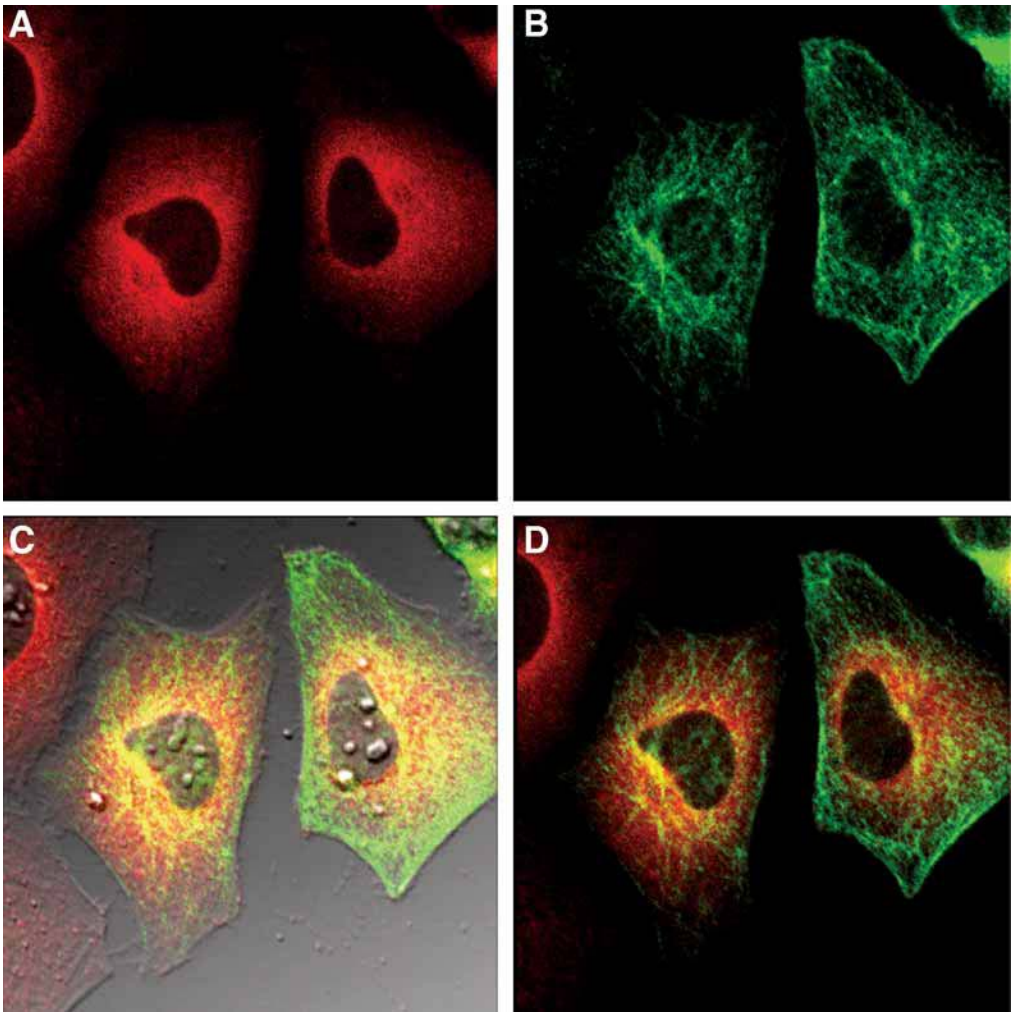

Fig. 7. Immunocytochemistry of the cells expressing the p65-HaloTag™ FLAG fusion protein and labeled with the HaloTag TMR ligand. HeLa cells transiently transfected with the vector encoding the p65-HaloTag FLAG fusion protein were labeled with 5 μ*M* HaloTag TMR Ligand. Cells were fixed with 3.7% paraformaldehyde and processed for immunocytochemistry using primary mouse Antiβ-III Tubulin Antibody (1μg/mL) and secondary goat antimouse IgG, conjugated with AlexaFluor™-488. Images were generated on an Olympus FV500 confocal microscope in sequential mode using appropriate filter sets for TMR, AlexaFluor™-488, or transmitted light. (**A**) TMR fluorescence. (**B**) AlexaFluor™-488 fluorescence. (**C**) Overlaid AlexaFluor™-488 and TMR fluorescence and transmitted light. (**D**) Overlaid AlexaFluor™-488 and TMR fluorescence.

3.4. Fluorescent Detection of HaloTag Protein Fusions in Living and Fixed Mammalian Cells, and by Protein Gel Electrophoresis

Although subcellular location and translocation of HaloTag protein fusions might be determined by fluorescence microscopy, the ability to resolve subcellular structures or fluorescently labeled proteins within specific cell compartments varies with instrument capabilities. We recommend using a confocal microscope with high numerical aperture objectives for achieving good results *(17,18)*.

Fluorescently labeled proteins can also be detected and quantified using fluorescence scanners, flow cytometry or fluorescence plate readers. Accurate quantification of the fluorescence could be complicated by a number of factors including low protein expression, instrument sensitivity, and quality of cell growth surfaces.

3.4.1. Fluorescent Detection of HaloTag Protein Fusions in Living Cells

1. Cells were imaged on a confocal microscope FV500 (Olympus, Japan) using a 488 nm Ar/Kr laser line or a 543- or 633-HeNe laser line. Scanning speed and laser intensity were adjusted to avoid photobleaching of the fluorophores and damage of the cells.
2. The cells labeled with the HaloTag-Coumarin Ligand were imaged on Olympus IX81epifluorescent microscope (Olympus, Japan) equipped with Chroma filter set no. 31,000 DAPI using Hg-lamp and an Orca CCD camera (Hamamatsu, Japan). The microscope was equipped with a microenvironmental chamber to maintain physiological conditions during long-term experiments.

3.4.2. Cell to Gel Analysis: Detection of HaloTag Fusion Proteins Using SDS-PAGE and a Fluorescence Imager

Because the HaloTag Ligand is held by a stable covalent bond, the fluorescently labeled HaloTag Protein can be boiled with sample buffer and resolved by SDS-PAGE without loss of the fluorescence signal. Analyzing labeled HaloTag Protein can be combined with other protein analysis techniques such as Western blotting. Our preliminary data indicate that this approach also can be used successfully to study posttranslational modification of the HaloTag Protein-based fusions (e.g., proteolytic cleavage, data not shown).

1. Cells transiently expressing the HaloTag-based fusion proteins and labeled with the HaloTag TMR Ligand were solubilized in a sample buffer, boiled for 5 min, and resolved on SDS-PAGE (4–20% gradient gels; BioWhittaker Molecular Applications).
2. Gels were analyzed on a fluorescence imager Typhoon 9400 (Hitachi, Japan) at an E_{ex}/E_{em} appropriate for TMR, were processed for Western blot analysis, or were stained with Coomassie blue (Promega).

3.4.3. Western Blot Analysis

1. Electrophoretic transfer of proteins to a nitrocellulose membrane (0.2 μm, Scheicher and Schuell, Germany) was carried out in transfer buffer for 2.0 h with a constant current of 80 mA (at 4°C) in Xcell II Blot module (Invitrogen).
2. The membranes were rinsed with TBST buffer and incubated in blocking solution for 30 min at room temperature or overnight at 4°C.
3. Membranes were washed with 50 mL of TBST buffer and incubated with anti-p65 antibody (dilution 1:5000), anti-IkB antibody (dilution 1:10,000), or anti-HaloTag antibody (dilution 1:50,000) for 45 min at room temperature.
4. The membranes were washed with TBST buffer (50 mL, 5 min, three times).
5. The membranes were then incubated with HRP-conjugated donkey antimouse IgG (30 min, room temperature) and then the washing procedure was repeated.
6. The proteins were visualized by the enhanced chemiluminescence system (Pharmacia-Amersham) according to the manufacturer's instructions. Levels of proteins were quantified using computer-assisted densitometry.

3.5. Conclusions

The HaloTag Interchangeable Labeling Technology provides a means for attaching small synthetic molecules onto a specific fusion protein within living mammalian cells. The synthetic molecules are attached covalently to the fusion protein through a short PEG-like tether capable of penetrating cellular membranes. The kinetics of binding are rapid, and the covalent bond securely and specifically locks the synthetic molecules in place. The molecules are retained on the fusion protein even under denaturing conditions, such as in fixed cells or in SDS electrophoresis.

The technology enables fusion proteins within cells to be labeled with a range of standard fluorophores for image analysis. This provides the benefits of allowing different colors to be used interchangeably on the same genetic construct or transgenic cells; of allowing conditional switching of the colors, thus enabling "pulse-chase" type experiments; and of providing a range of colors in fixed cells, in which other methods tend to be inefficient or unstable. Because the fluorophore can also be exchanged with an affinity handle, such as biotin, the fusion proteins might also be captured from cells for in vitro analysis. Fusion proteins might also be specifically and stably attached directly onto surfaces containing the reactive tether without requiring preceding sample purification.

The unrestricted architecture of the binding interaction opens the possibility for incorporating other types of synthetic molecules. Examples might include fluorophores that can respond to subcellular microenvironments or indicate physiological changes occurring within cells. The synthetic molecules might also mediate interactions with other proteins within the cells, or trigger specific subcellular events. The efficiency and specificity of the interaction even within the extreme complexity of living cells might offer new technical opportunities, in which current alternatives have not been sufficient.

4. Notes

1. The level of the HaloTag Protein expression depends on many factors including cell type, efficiency of transfection, type of promoter, and protein coding sequence. Transfection can be toxic to cells, which frequently correlates with the transfection efficiency. Balance between sufficient protein expression, transfection efficiency, and low toxicity is essential for generating reliable data. Cells should be actively proliferating. The recommended cell density for most cell lines at transfection time is approx 80–90% confluency. Preliminary experiments should be done to optimize cell density, amount of DNA, and transfection reagent for transient transfection. Efficiency of transfection might be affected by the specific cell line, cell culture and transfection conditions, and specific DNA constructs.
2. The concentration of the HaloTag Ligands, and cell labeling and washing protocols, should be optimized for different cell lines and different applications. HaloTag TMR, HaloTag diAcFAM, and HaloTag Biotin Ligands have exhibited no detectable toxicity or morphological side effects at recommended labeling conditions in the cell lines tested (HeLa, CHO-K1). HaloTag Ligands can be added to serum-containing medium directly. The HaloTag TMR and HaloTag Biotin Ligands can be premixed with medium. In contrast, the HaloTag diAcFAM Ligand must be mixed with media immediately before adding it to the cells to avoid premature hydrolysis of the diacetyl groups by serum esterases. The HaloTag diAcFAM Ligand can cross-cellular membranes, but the deacetylated FAM derivative cannot. Because fluorescent dyes are light sensitive, light exposure of the cells should be avoided during ligand loading and washing procedures.

Appendix—HaloTag Mechanism

The native hydrolase is a monomer with MW approx 33 kDa. The activity of enzyme cleaves carbon–halogen bonds in aliphatic halogenated compounds involving a hydrolytic triad at the active site.

$$R - Cl + Enz + HOH \rightarrow R - Enz + Cl- + HOH \rightarrow R- OH + Enz + H^{+} + Cl-$$

In the reaction catalyzed by the native enzyme, an enzyme–substrate complex is formed by a nucleophilic attack involving Asp106 and the formation of an ester intermediate; His272 activates H_2O that hydrolyzes this intermediate, releasing product from the catalytic center (**Fig. 8**; **refs. *19–21***). A point mutation in the gene resulting in a His272Phe substitution impairs the hydrolysis step, leading to formation of a covalent bond between protein and ligand containing the functional reporters (**Fig. 9**).

The amino acid sequence of the protein was further optimized to provide better access to the active site by the ligand. These changes result in a dramatic increase in the kinetics of ligand

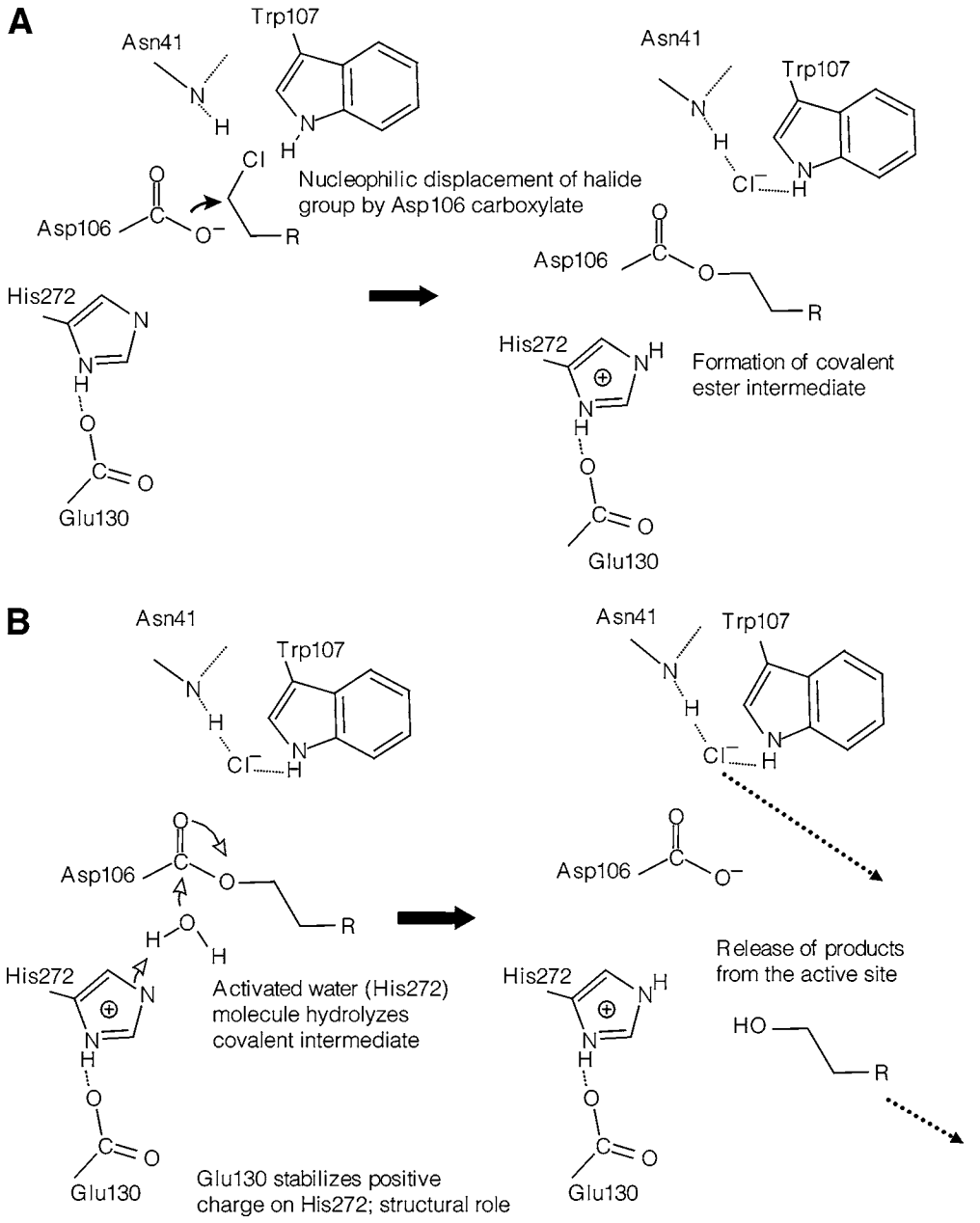

Fig. 8. The catalytic mechanism of wild-type hydrolase.

binding rate by several 1000-fold, leading to almost immediate binding for HaloTag TMR Ligand binding to GST-HaloTag Protein fusion.

Acknowledgments

The HaloTag technology presented in this chapter represents the creative insights and efforts from many scientists. The authors wish to acknowledge a long list of contributors from Promega Corporation and Promega Biosciences for this accomplishment.

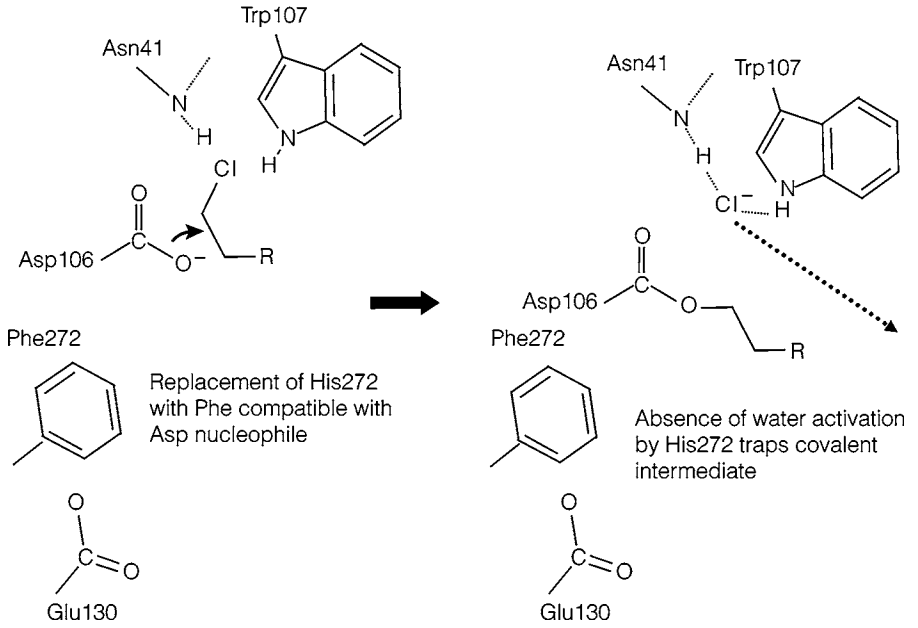

Fig. 9. The engineered protein encoded by the HaloTag™ pHT2 vector includes the His272Phe substitution, which is critical for covalent bond formation.

References

1. Los, G. et al. (2005) HaloTag™ interchangeable labeling technology for cell imaging and protein capture. *Cell Notes* **11,** 2–6.
2. Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39(3 Part 2),** 499–509.
3. Ghosh, S., May, M. J., and Kopp, E. B. (1998) NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–260.
4. Karin, M., Yamamoto, Y., and Wang, Q. M. (2004) The IKK NF-kappa B system: a treasure trove for drug development. *Nature Rev.* **3,** 17–26.
5. Burnstein, E. and Duckett, C. S. (2003) Dying for NF-kappaB? Control of cell death by transcriptional regulation of the apoptotic machinery. *Curr. Opin. Cell Biol.* **15,** 732–737.
6. Schmid, J. A., Birbach, A., Hofer-Warbinek, R., et al. (2000) Dynamics of NF kappa B and Ikappa Balpha studied with green fluorescent protein (GFP) fusion proteins. Investigation of GFP-p65 binding to DNa by fluorescence resonance energy transfer. *J Biol. Chem.* **275 (22),** 17,035–17,042.
7. Freshney, R. I. (1986) *Animal Cell Culture: A Practical Approach*, IRL Press, Oxford, UK.
8. Ausubel, F. M. et al. (1994) *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley-Interscience, New York.
9. Felgner, P. L., Holm, M., and Chan, H. (1989) Cationic liposome mediated transfection. *Proc. West. Pharmacol. Soc.* **32,** 115–216.
10. Felgner, P. L. and Ringold, G. M. (1989) Cationic liposome-mediated transfection. *Nature* **337,** 387–388.
11. Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell Biol.* **7,** 2745–2752.
12. Wigler, M. et al. (1977) Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11,** 223–232.
13. Chu, G., Hayakawa H., and Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucl. Acids Res.* **15,** 131–226.
14. Shikegawa, K. and Dower, W. J. (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. *Biotechniques* **6,** 742–751.

15. Ramezani, A. and Hawley, R. G. (2002) Overview of the HIV-1 lentiviral vector system, in *Current Protocols in Molecular Biology,* (Ausubel, F.M. et al., ed.), John Wiley and Sons, Inc., Hoboken, NJ, pp. 16.21.1–16.21.15.
16. Moss, B. and Earl, P. (1998) Overview of the vaccinia virus expression system, in *Current Protocols in Molecular Biology,* (Ausubel, F.M. et al., ed.) John Wiley and Sons, Inc., Hoboken, NJ, pp. 16.15.1–16.15.5.
17. Herman, B. (2001) *Fluorescence Microscopy*, 2nd ed., Springer-Verlag, New York.
18. Perassamy, A., ed. (2001) *Methods in Cellular Imaging,* Oxford University Press, New York.
19. Kulakova, A. N., Larkin, M. J., and Kulakov, L. A. (1997) The plasmid-located haloalkane dehalogenase gene from Rhodococcus rhodochrous NCIMB 13064. *Microbiology* **143,** 109–115.
20. Schindler, J. F. et al. (1999) Haloalkane dehalogenase: steady-state kinetics and halide inhibition. *Biochemistry* **38,** 5772–5778.
21. Newman, J. et al. (1999) Haloalkane dehalogenase: structure of a *Rhodococcus* enzyme. *Biochemistry* **38,** 16,105–16,114.