



# HaloTag® Protein: A Novel Reporter Protein for Human Neural Stem Cells

**ABSTRACT** Stem cells and related proteins are the focus of many scientific studies. The ability to label specific proteins within stem cells may provide important information about protein dynamics and function. Here we use a technology to covalently tether different wavelength fluorophores to a specially designed reporter, the HaloTag® protein. The HaloTag® protein, either alone or fused to a protein of interest, was expressed in human neural stem cells (hNSC) and successfully labeled with several HaloTag® Ligands. The different colored cell-impermeant and cell-permeant fluorophores showed spatial separation and real-time translocation of membrane and internal protein pools in live hNSC. In addition, the HaloTag® protein continued to be expressed after hNSC differentiation into neurons and astrocytes. Thus, the HaloTag® technology can be used to study proteins of interest, and as such, represents a new research tool to further reveal protein biology in stem cells.

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**Stem cells** are being used to study basic biology, to screen drugs, and to model and potentially treat human diseases.

## INTRODUCTION

Specific labeling of proteins in live cells may provide details about their functions and dynamics within complex intracellular environments. The development of new methods for labeling proteins by genetic fusion is expanding the understanding of cellular biology. Stem cells are being used to study basic biology, to screen drugs, and to model and potentially treat human diseases (1). Here, we use the HaloTag® technology<sup>(a-c)</sup> in human neural stem cells (hNSC) to visualize the HaloTag® reporter protein alone and fused to proteins of interest.

## HALOTAG® TECHNOLOGY OVERVIEW

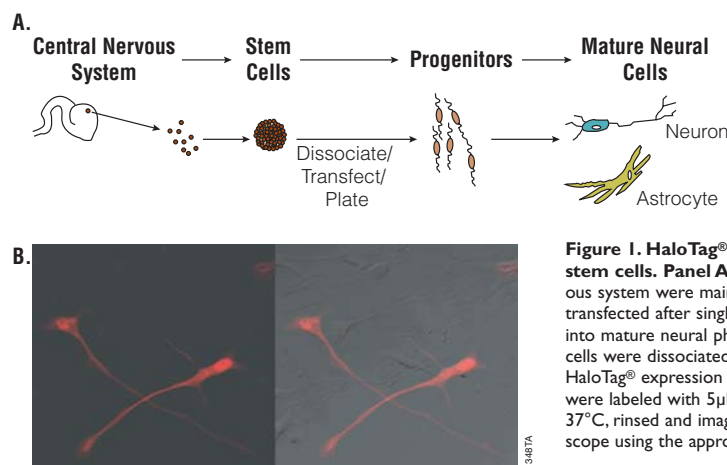
The HaloTag® technology permits tethering of different wavelength fluorophores to a reporter protein with a single genetic construct (2). The HaloTag® reporter protein is an engineered, catalytically inactive derivative of a bacterial hydrolase that forms a covalent bond with HaloTag® ligands. The HaloTag®

ligands contain two crucial components: a common HaloTag® reactive linker that forms a covalent bond with the HaloTag® protein and a functional reporter (e.g., a fluorescent dye) or an affinity tag (e.g., biotin).

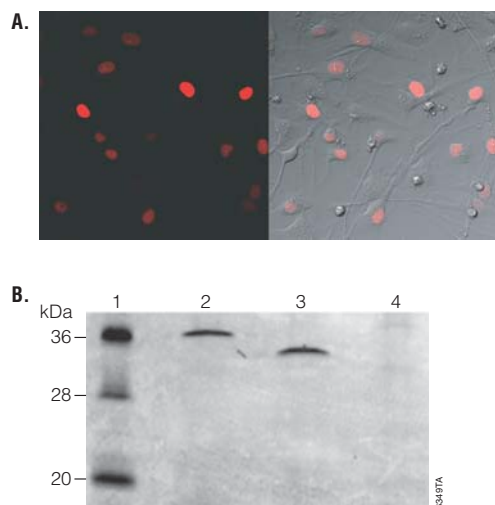
## HALOTAG® EXPRESSION AND LABELING IN HUMAN NEURAL STEM CELLS

We used stem cells derived from the developing human central nervous system, which are then maintained as spheres of cells termed neurospheres (Figure 1, Panel A; 3). Neurospheres can be transfected after single-cell dissociation, then plated and differentiated into mature neural phenotypes.

Previously, we showed expression of the HaloTag® protein in a variety of mammalian cell lines (2), and now we have expressed the HaloTag® reporter protein in hNSC. Cells appeared healthy and had normal morphology after expressing the HaloTag® reporter protein and labeling with HaloTag® TMR Ligand (Figure 1, Panel B).



**Figure 1. HaloTag® reporter protein expression in neural stem cells. Panel A.** Stem cells derived from the central nervous system were maintained as neurospheres, which can be transfected after single-cell dissociation, plated and differentiated into mature neural phenotypes. **Panel B.** Human neural stem cells were dissociated to single cells and transfected with the HaloTag® expression vector. Forty-eight hours after plating, cells were labeled with 5µM HaloTag® TMR Ligand for 15 minutes at 37°C, rinsed and imaged with an Olympus FV500 confocal microscope using the appropriate filter set.



**Figure 2. HaloTag<sup>®</sup> protein subcellular localization in neural stem cells.** Panel A. Cells expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein were labeled with 5 μM HaloTag<sup>®</sup> TMR Ligand for 15 minutes at 37°C, rinsed and imaged with an Olympus FV500 confocal microscope using the appropriate filter set. Panel B. Cells expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein (lane 2), HaloTag<sup>®</sup> protein alone (lane 3), or nontransfected (lane 4) were labeled with the HaloTag<sup>®</sup> TMR Ligand, lysed and run on SDS-PAGE with ladder (lane 1).

**HALOTAG<sup>®</sup> SUBCELLULAR LOCALIZATION IN HUMAN NEURAL STEM CELLS**

The HaloTag<sup>®</sup> protein can be expressed alone or fused to a protein or target sequence that directs the fusion protein to a specific subcellular compartment. We expressed three repeats of a nuclear localization sequence (NLS)<sub>3</sub> from the SV40 large T-antigen fused to the C-terminus of the HaloTag<sup>®</sup> protein (4,5). Live-cell imaging of hNSC labeled with HaloTag<sup>®</sup> TMR Ligand showed HaloTag<sup>®</sup>-(NLS)<sub>3</sub> protein localized solely to the nucleus, and this fusion protein was expressed for up to 120 hours following transfection (Figure 2, Panel A).

The HaloTag<sup>®</sup> protein labeled with HaloTag<sup>®</sup> ligands can be resolved by electrophoresis without detectable loss of the fluorescent signal, permitting the analysis of HaloTag<sup>®</sup> proteins in vitro. Human NSC expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion, HaloTag<sup>®</sup> protein alone or untransfected were labeled with the HaloTag<sup>®</sup> TMR Ligand, lysed and analyzed by SDS-PAGE. Results showed a clear band for the HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein and a lower molecular weight band for the HaloTag<sup>®</sup> protein alone (Figure 2, Panel B). No protein band was detected in untransfected hNSC, showing the specificity of the HaloTag<sup>®</sup> technology.

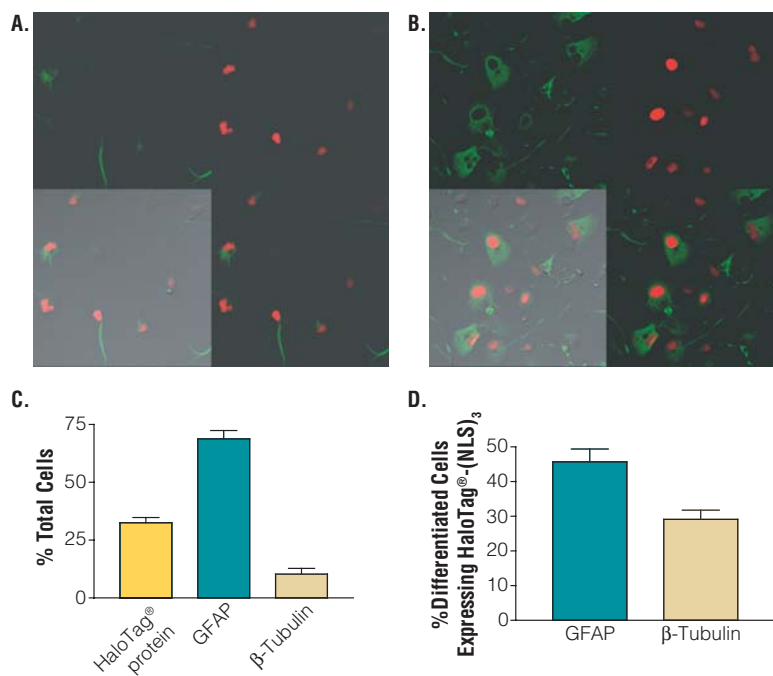
**HALOTAG<sup>®</sup> EXPRESSION IN DIFFERENTIATED HUMAN NEURAL STEM CELLS**

The HaloTag<sup>®</sup> technology allows multiplexing experiments that employ live-cell labeling followed by immunocytochemistry (ICC) because the HaloTag<sup>®</sup> ligands retain their fluorescent properties after fixation. We used this feature

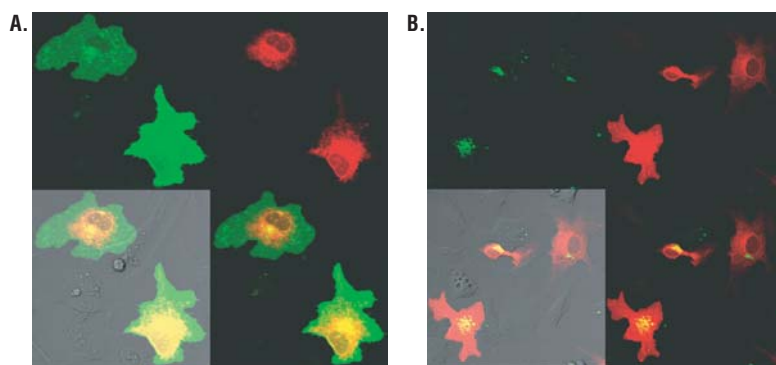
to assess whether hNSC differentiated into neurons and astrocytes continue to express the HaloTag<sup>®</sup> fusion protein. Live hNSC expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein were labeled with the HaloTag<sup>®</sup> TMR Ligand, then fixed and processed for ICC using antibodies against β-tubulin, a neuronal marker, and glial fibrillary acidic protein (GFAP), an astrocytic marker (Figure 3, Panels A and B). The results showed that the HaloTag<sup>®</sup> protein is still expressed after the transfected hNSC have differentiated into neurons and astrocytes.

To complement the qualitative ICC results, we quantified the percent total cells transfected with HaloTag<sup>®</sup>-(NLS)<sub>3</sub> protein and the percent total cells that differentiated into neurons and astrocytes. From the total number of plated and transfected hNSC, approximately 30% were expressing HaloTag<sup>®</sup> protein (based on HaloTag<sup>®</sup> TMR Ligand labeling), 70% were astrocytes (based on GFAP labeling) and 20% were neurons (based on β-tubulin labeling; Figure 3, Panel C). The remaining cells likely represent undifferentiated neural progenitor cells. Human NSC appear to tolerate the HaloTag<sup>®</sup> protein since their differentiation rates are consistent with previous reports (3). Within differentiated cells expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein, approximately 45% were astrocytes, and 30% were neurons (Figure 3, Panel D). This difference was not significant, suggesting that the HaloTag<sup>®</sup> reporter protein had no selective effects on cell type survival or differentiation.

**The HaloTag<sup>®</sup> protein is still expressed after the transfected human NSC have differentiated into neurons and astrocytes.**



**Figure 3. HaloTag<sup>®</sup> protein expression in differentiated neural stem cells.** Cells expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein were labeled as described for Figure 2 and then fixed for ICC with antibodies against β-tubulin (mouse, 1:2,000, Sigma; Panel A) or GFAP (rabbit, 1:1,000, Dako; Panel B) followed by Alexa Fluor<sup>®</sup> 488-conjugated antibodies (1:1,000, Molecular Probes). Images were taken with an Olympus FV500 confocal microscope using the appropriate filter set. Panel C. The percent of plated cells expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> protein, percent astrocytes and percent neurons. Panel D. The percent of astrocytes and neurons expressing HaloTag<sup>®</sup>-(NLS)<sub>3</sub> fusion protein.



**Figure 4. HaloTag® spatial and temporal separation in neural stem cells. Panel A.** Cells expressing  $\beta 1$ Int-HaloTag® fusion were pulsed with  $5\mu\text{M}$  HaloTag® Alexa Fluor® 488 Ligand for 15 minutes at  $37^\circ\text{C}$ , chased with cell-permeant  $5\mu\text{M}$  HaloTag® TMR Ligand for 15 minutes at  $37^\circ\text{C}$ , rinsed and imaged with Olympus FV500 confocal microscope in sequential mode using appropriate filter sets. **Panel B.** Twelve hours after cell labeling, imaging showed the differentially labeled proteins as they moved from the cytoplasm to the membrane and as they internalized from the membrane.

#### HALOTAG® PROTEIN SPATIAL AND TEMPORAL SEPARATION IN HUMAN NEURAL STEM CELLS

In mammalian cell lines, we have shown that the HaloTag® reporter protein fused to a truncated  $\beta 1$  integrin was expressed on the cell surface and could be labeled with the cell-impermeant fluorescent HaloTag® Alexa Fluor® 488<sup>(a-c)</sup> Ligand (6). We show that hNSC can also express the HaloTag® reporter protein fused to a truncated integrin. Human NSC were first labeled with the cell-impermeant HaloTag® Alexa Fluor® 488 Ligand and then labeled with the cell-permeant HaloTag® TMR Ligand. Live-cell imaging showed the  $\beta 1$ Int-HaloTag® protein in two distinct protein pools with the cell-surface HaloTag® protein labeled by the HaloTag® Alexa Fluor® 488 Ligand and the intracellular HaloTag® protein labeled by the HaloTag® TMR Ligand (Figure 4, Panel A). Expression of the  $\beta 1$ Int-HaloTag® protein and labeling with the HaloTag® Alexa Fluor® 488 Ligand caused no detectable morphological changes in hNSC.

Labeling of  $\beta 1$ Int-HaloTag® fusion protein with a cell-impermeant HaloTag® Alexa Fluor® 488 Ligand followed by a cell-permeant HaloTag® TMR Ligand can also temporally distinguish two protein pools. Twelve hours after labeling of hNSC expressing the  $\beta 1$ Int-HaloTag® fusion, live-cell imaging showed translocation of the differentially labeled protein pools, as the cell-impermeant green surface pool was internalized from the membrane and the cell-permeant red internal pool was trafficked to the surface (Figure 4, Panel B).

#### CONCLUSION

The HaloTag® technology is a powerful tool to study proteins in live cells. Using neural stem cells, we showed that the HaloTag® technology can be used to follow labeled proteins in hNSC. We demonstrated hNSC can express the HaloTag® reporter protein alone and fused to proteins of interest. In addition, HaloTag®-expressing

hNSC can be successfully labeled by multiple HaloTag® ligands. The HaloTag® protein was well-tolerated by hNSC and continued to be expressed following differentiation into neurons and astrocytes. Fusion to a NLS target sequence directed the HaloTag® protein to the proper subcellular compartment, and fusion to a truncated integrin provided surface-displayed HaloTag® protein in hNSC. Labeling of  $\beta 1$ Int-HaloTag® fusion with cell-impermeant and permeant HaloTag® ligands showed both spatial separation of protein pools and translocation of surface and intracellular protein pools in hNSC. In conclusion, the HaloTag® technology provides a powerful and flexible tool to examine intracellular and membrane proteins of interest to stem cell biology.

#### REFERENCES

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Ordering information is available on pages 27–28 or online at: [www.promega.com/halotagordering/](http://www.promega.com/halotagordering/)

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