PERFORM MULTICOLOR LIVE- AND FIXED-CELL IMAGING APPLICATIONS WITH THE HALOTAG™ INTERCHANGEABLE LABELING TECHNOLOGY

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Here we describe imaging experiments using the HaloTag™ Protein and Ligands. The HaloTag™ Technology provides users flexibility in designing multicolor imaging experiments involving intrinsically fluorescent proteins (IFPs) and immunocytochemistry.

Introduction

HaloTag™ Interchangeable Labeling Technology[a–d] is useful for imaging, capturing or immobilizing fusion proteins. The HaloTag™ Protein binds rapidly and specifically to chloroalkane ligands that have been modified to carry a variety of functional chemical tags. The interchangeable nature of the chemical tag allows users to label a single fusion construct with either a red, green or blue (under development) fluorophore for imaging or with a biotinylated ligand for downstream applications using streptavidin reagents (1). This availability of multiple tags can simplify multiplexing applications.

Multiplexing in Live-Cell Imaging Experiments

With the discovery of intrinsically fluorescent proteins (IFPs) such as the green fluorescent protein (GFP) and the subsequent creation of a full-color spectrum of these IFPs, researchers can now fuse a protein of interest to IFPs with a variety of properties. However, multiplexing with IFPs requires careful consideration of fluorophore options prior to initiating the experiments. Before cloning, researchers must select an IFP with a spectral profile compatible with filter sets available to the researcher. The chosen IFP gene is fused to a gene of interest, expressed, and then visualized.

> These data highlight the flexibility associated with using the HaloTag™ Technology in combination with an IFP.

Multicolor imaging may be carried out using an IFP fusion protein and a second fluorophore with a different emission wavelength. The latter reagent can be a counterstaining dye (for example a DNA-binding dye) that provides a positional reference point within the cell (the nucleus). Thus, with a second set of fluorescence filters, localizing the fusion protein with reference to a subcellular structure in living cells is possible. If the IFP and the dye are stable to fixation and detergents, a third fluorophore can be introduced through the use of immunocytochemical staining. However, the third fluorophore must be spectrally distinct from the IFP. For example if a researcher chooses a "green" IFP, this choice generally prohibits use of green counterstains or secondary antibodies conjugated to green fluorophores.

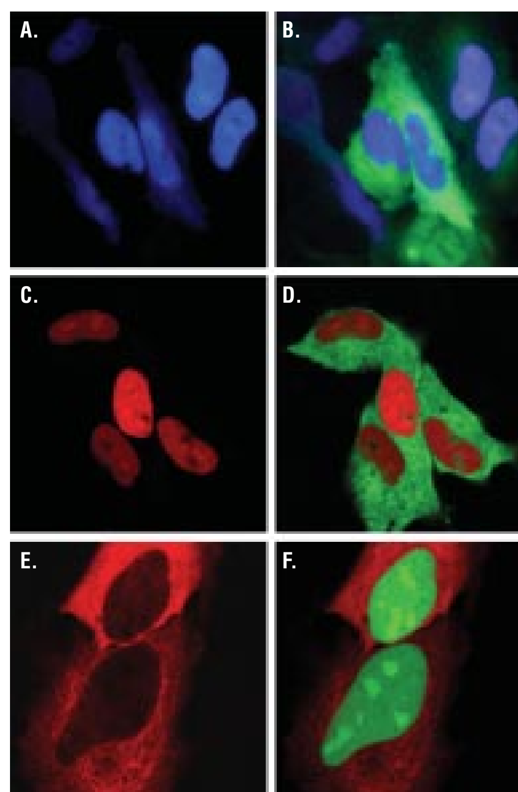

Figure 1. Targeting HaloTag™ and hMGFP reporters to the nucleus or cytoplasm of live cells. HeLa cells were transiently co-transfected with either HaloTag™-$(NLS)_3$ plus hMGFP-α-tubulin (**Panels A–D**) or HaloTag™-α-tubulin plus hMGFP-$(NLS)_3$ (**Panels E and F**). Twenty-four hours later, cells expressing HaloTag™-$(NLS)_3$ were incubated with either 25µM blue ligand (under development, **Panels A and B**) or 5µM HaloTag™ TMR Ligand (**Panels C and D**) for 15 minutes; cells expressing HaloTag™-α-tubulin were incubated with 5µM HaloTag™ TMR Ligand for 15 minutes at 37°C/5% CO_2 (**Panels E and F**). Cells were washed and incubated for 30 minutes. In **Panels A and B**, cells were imaged with an Olympus IX81 epifluorescent microscope equipped with Chroma filter sets (#31000 DAPI for the blue ligand and #41001 FITC for hMGFP), a Hamamatsu Orca CCD camera and environmental controls. Images in **Panels C–F** were captured with the Olympus FV500 confocal attachment with sequential two-laser scanning and filters appropriate for TMR and FITC fluorescence.

Multiplexing Live- and Fixed-Cell Imaging Experiments

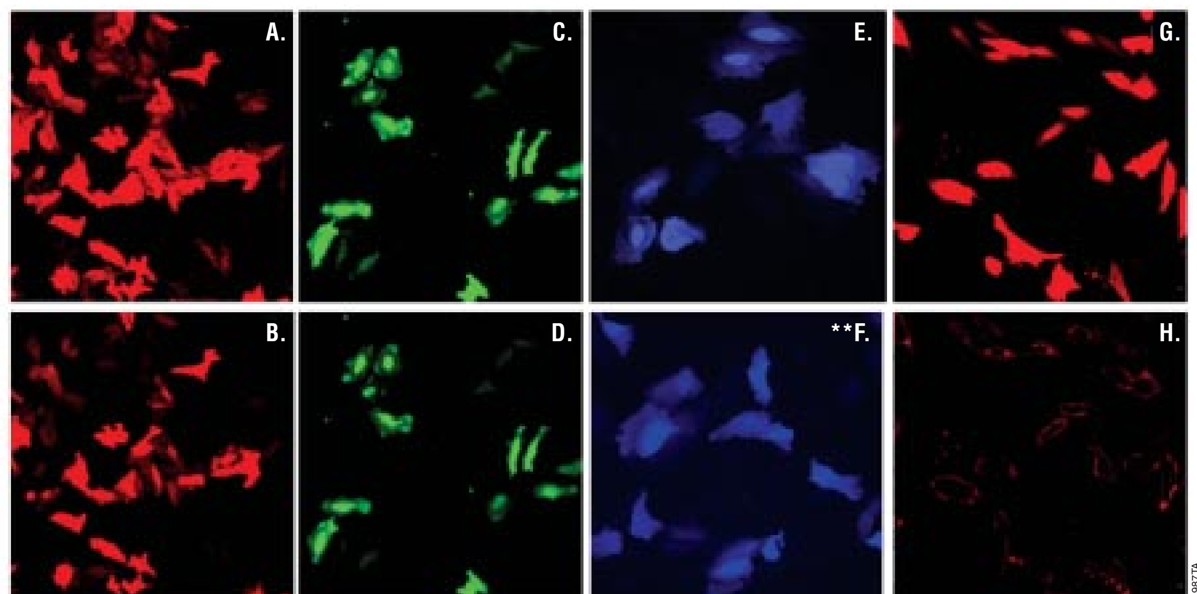

Figure 2. HaloTag™ Ligands withstand fixation. HeLa cells were transiently transfected with either the HaloTag™ expression vector or a red IFP (DsRed; BD BioScience Clonetech Cat.# 6974-1). Twenty-four hours later, cells expressing the HaloTag™ Protein were labeled with HaloTag™ TMR Ligand (5µM, **Panels A and B**), HaloTag™ diAcFAM Ligand (10µM, **Panels C and D**), or the blue ligand (currently under development; 25µM, **Panels E and F)** for 15 minutes at 37°C/5% CO_2. Unbound ligand was washed from cells, and cells were incubated for 30 minutes. Live cells were imaged and then cells were fixed with warm 4% paraformaldehyde plus 0.4M sucrose in PBS for 10 minutes. Fixed cells were imaged, again using identical settings for fluorophore excitation and detection of emission light. **Panel G** shows staining of live cells expressing the multimeric DsRed IFP. **Panel H** shows the same cells after fixation. **Panels A,C,E and G** represent cells imaged before fixation; **Panels B,D,F and H** were imaged after fixation. ****Note:** Images in **Panels E and F** were captured with a CCD camera and represent different fields of view within the same culture well. All other images were captured with a confocal microscope and show identical fields of view before and after fixation.

The HaloTag™ Technology can simplify multicolor experiments. Since the HaloTag™ Protein is not an IFP, the choice of fluorescent labels, including secondary and tertiary fluorophores, can be made after creating the HaloTag™ fusion protein. This feature allows more flexibility in the choice of fluorophores.

To demonstrate this flexibility in a multicolor imaging experiment, two sets of expression vectors were created to deliver either the HaloTag™ reporter protein or humanized Monster Green® Fluorescent Protein (hMGFP; Cat.# E6421) to different subcellular compartments. In the first experiment, three repeats of a nuclear localization sequence [$(NLS)_3$] were added to the C-terminus of both the HaloTag™ Protein and hMGFP. We also created proteins targeted to the cytoplasm by fusing the α-tubulin gene to the C-terminus of each reporter protein. HeLa cells were transiently co-transfected with either HaloTag™-$(NLS)_3$ plus hMGFP-α-tubulin or HaloTag™-α-tubulin plus hMGFP-$(NLS)_3$. Cells were incubated with either the HaloTag™-TMR Ligand or a blue ligand (currently under development), and live-cell images were captured (Figure 1).

The HaloTag™ Ligand labeling was localized to the nucleus in HaloTag™-$(NLS)_3$-transfected cells. This pattern was present in cells containing both the blue ligand- and TMR Ligand-labeled HaloTag™ fusion proteins (Figure 1, Panels A and C). Not surprisingly, a low level of cytoplasmic HaloTag™ signal was also detected in some cells (Figure 1, Panel A), which may represent protein being translocated to the nucleus. In contrast, in both TMR and blue ligand-labeled cells, the intrinsic green fluorescence of the co-transfected hMGFP-α-tubulin fusion protein was confined to the cytoplasm (Figure 1, Panels B and D). The HaloTag™-α-tubulin fusion protein was also localized to the cytoplasm, as demonstrated by labeling with the TMR Ligand (Figure 1, Panel E). Thus, both α-tubulin fusion reporters were expressed in the expected location for the native cytoskeletal protein. The localized intrinsic green fluorescence of the expressed hMGFP-$(NLS)_3$ construct (Figure 1, Panel F) confirms that the hMGFP fusion reporter was also successfully targeted to the nucleus.

These data highlight the flexibility associated with using the HaloTag™ Technology in combination with an IFP. They demonstrate simultaneous targeting to separate subcellular compartments with spatial resolution of two reporters. The HaloTag™ Technology offers options for choosing the properties of the second fluorophore to achieve the best spatial and spectral resolution.

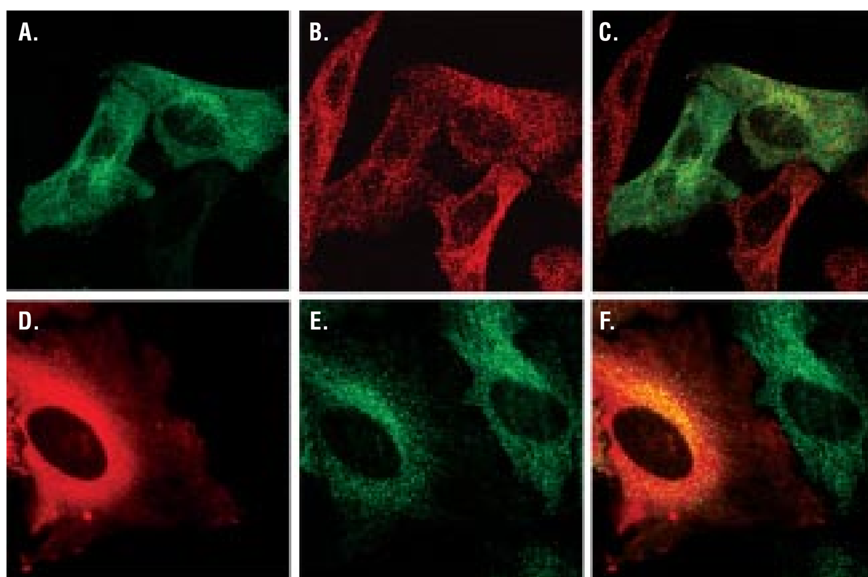

Figure 3. Multiplexing HaloTag™ and Immunocytochemistry. HeLa cells were transfected with HaloTag™-α-tubulin, labeled with HaloTag™ diAcFAM (**Panels A–C**) or TMR Ligands (**Panels D–F**), washed, and fixed as described in Figure 2. Cells were permeabilized with 0.1% Triton® X-100 and immunolabeled with Anti-βIII Tubulin mAb (1:5,000 dilution, Cat.# G7121). Cells were incubated with 1:500 dilutions of Alexa Fluor™ 488-conjugated secondary antibody (**Panels A–C**) or Cy®3-conjugated secondary antibody (**Panels D–F.**) **Panels A and D** show cells labeled with the HaloTag™ Ligands only; Panel A, diAcFAM Ligand; Panel B, TMR Ligand. **Panels B and E** show labeling for βIII tubulin. **Panels C and F** show double staining for the HaloTag™ Protein and βIII tubulin.

Multiplexing Live- and Fixed-Cell Imaging Experiments

To address the possibility of performing multiplexing experiments that employ immunocytochemistry (ICC), we first sought to demonstrate that HaloTag™ Ligands would retain their fluorescent properties after fixation. For this set of experiments, live HeLa cells expressing the HaloTag™ Protein were labeled with one of three ligands, imaged, then fixed and imaged again. In parallel, a commonly used multimeric red IFP (pDsRed2-Cl, BD Bioscience Clonetech Cat.# 6984-1) was expressed, and its fluorescent signal was monitored before and after fixation (Figure 2).

For each of the HaloTag™ Ligands, nearly all of the fluorescent signal was retained after fixation. Conversely, fluorescence of the multimeric red IFP (DsRed) was dramatically diminished after treatment with paraformaldehyde. Disruption of the multimeric structure upon exposure to aldehydes may explain the instability and loss of fluorescence in this assay. Conversely, once ligand is bound to the HaloTag™ Protein, the fluorescent properties of the molecule are independent of the structure of the HaloTag™ monomeric protein, and thus fluorescence withstands fixation.

Multiplexing HaloTag™ Protein Labeling with ICC

These data, and previously reported data using the HaloTag™ TMR Ligand (reference 1, Figure 8), suggested that it would be possible to multiplex HaloTag™ labeling with ICC staining techniques. Therefore, we labeled cells expressing HaloTag™-α-tubulin with the TMR Ligand or the diAcFAM Ligand and processed the cells for ICC using a primary antibody against βIII-tubulin (Cat.# G7121) and Alexa Fluor™ 488- (Molecular Probes Cat.# A11001) or Cy®3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Cat.# 751-165-150), respectively (Figure 3).

All HeLa cells expressed βIII-tubulin in the cytoplasm. The HaloTag™-α-tubulin reporter was localized to the cytoplasm in a subpopulation of transfected cells. This observation was similar for both fluorophore combinations, including the combination of TMR and Alexa Fluor™ 488 fluorescence or diAcFAM and Cy®3 fluorescence. The analogous combination of a red IFP reporter (DsRed) and an Alexa Fluor™ 488 secondary antibody did not produce the desired dual-color image in this assay because of instability following fixation (data not shown). These observations demonstrate the utility of two-color imaging using HaloTag™ and immunocytochemistry (ICC) to label distinct cytoplasmic proteins.

Summary

The data provide two examples that demonstrate the flexibility of the HaloTag™ Technology in two-color imaging. In live-cell co-transfections with an IFP, the HaloTag™ Technology simplifies choices in fluorophore selection. The latter example, involving fixed cells and ICC, demonstrates further flexibility not only in selecting fluorophores but also in coupling with alternative imaging technologies, in particular fixed-cell ICC. Finally, we have designed ligands with new functionalities to expand the interchangeable properties of the HaloTag™ Technology. These ligands may carry new colors for imaging or affinity handles for capturing or immobilizing fusion proteins. These features expand the repertoire of multiplexing applications possible for each HaloTag™ fusion protein, reducing the requirement to produce multiple genetic constructions to express reporter proteins. ■

Reference

1. Los, G.V. *et al.* (2005) *Cell Notes* **11**, 2–6.

Protocol

HaloTag™ Interchangeable Labeling Technology Technical Manual #TM260
(www.promega.com/tbs/tm260/tm260.html)

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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3rd Symposium on Biological Imaging
Multidimensional Biological Imaging: Approaches and Innovations

The W.M. Keck Laboratory for Biological Imaging and the Laboratory for Optical and Computational Instrumentation at the University of Wisconsin-Madison, in cooperation with Promega Corporation, will present the 3rd Symposium on Biological Imaging. The symposium will be held in Madison, WI, at the BioPharmaceutical Technology Center on Friday, September 9, 2005.

The theme of this year's symposium is *Multidimensional Biological Imaging: Approaches and Innovations.*

The symposium will present recent advances in multidimensional imaging across a broad spectrum of biology. Lectures and poster presentations will highlight how newly developed imaging technology can be applied in solving a variety of biological problems, ranging from imaging at nanoscale resolution to capturing large-scale images of intact tissues noninvasively. Topics include: total internal reflection (TIRF) microscopy, fluorescence resonance energy transfer (FRET), whole animal molecular imaging, protein tracking with GFP, nanoscale fluorescence microscopy, multidimensional image analysis, and near-infrared diffuse optical imaging. In addition, the symposium will feature interactive workshops on fluorescence lifetime imaging microscopy (FLIM) presented by Becker and Hickl, and software-based methods for image acquisition and analysis presented by Molecular Devices Corporation.

Stefan Hell from the Department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry, Goettingen, will give the keynote address. Professor Hell will describe techniques recently developed in his laboratory that go beyond the diffraction limit of visible light, extending the resolution of fluorescence microsopy to nanoscale imaging. Lectures will also be given by Wolfhard Almers, Vollum Institute; Kevin Eliceiri, UW-Madison; Sanjiv Sam Gambhir, Stanford University; Jennifer Lippincott-Schwartz, N.I.H.; Badrinath Roysam, R.P.I.; Ted Salmon, University of North Carolina; and Bruce Tromberg, University of California, Irvine.

For further information, please see:
www.keck.bioimaging.wisc.edu/symposium05.htm

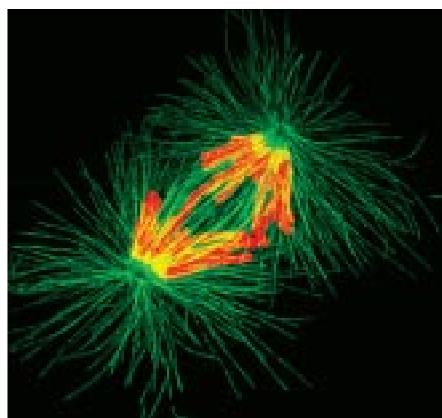
Image courtesy of Dr. Ted Salmon.