Assessing the Effectiveness of Self-Labeling Protein HaloTag for Imaging in Zebrafish Embryos

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Bridge UnderGrad Science (BUGS) ***Summer Research Program***

USC University of Southern California

Abstract

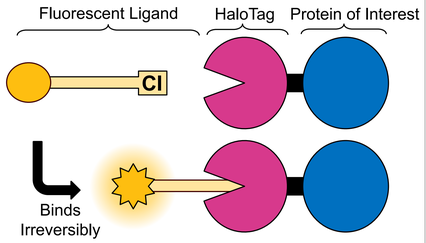
Zebrafish (*Danio rerio)* are a useful model organism for imaging due to their similarities to humans, embryo transparency, and external fertilization. HaloTag is a useful self-labeling protein tagging system that is used for the imaging of proteins *in vivo*. The studies demonstrating the use of HaloTag in zebrafish embryos focus on earlier stages of development. The development of the midbrain and hindbrain, which are useful for the study of Alzheimer's, occurs later, after 24 hours post fertilization (hpf). Studying the processing and movement of certain proteins in these regions–such as amyloid beta–helps to understand protein clearance and Alzheimer's. **HaloTag has strong potential as a tool for fluorescence microscopy in zebrafish to understand disease.**

Here we create an expression construct from HaloTag[9] using the pCS2+ vector to drive the transcription of HaloTag[9] mRNA. This mRNA is injected into single-celled embryos in order to drive the expression of HaloTag in zebrafish embryos. We imaged zebrafish at least 24hpf. To determine the ideal concentration of fluorescent ligand (Oregon Green 488 dye), four different concentrations of dye are tested in wildtype zebrafish. We found that there is no significant difference between the brightness of zebrafish with different concentrations of dye. The zebrafish injected with HaloTag and stained with dye were significantly brighter than those injected with HaloTag without dye or those with only dye. A Nuclear Localization Signal (NLS) version was also tested and was much brighter than regular HaloTag in the nuclei.

Our imaging results indicate HaloTag can be a useful tool for imaging proteins, especially those in extracellular space such as the amyloid beta or tau proteins which play a major role in Alzheimer's Disease pathogenesis.

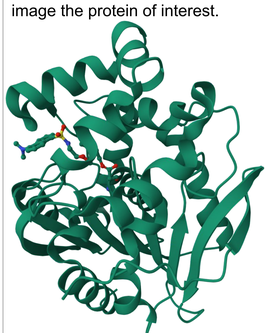
Background: HaloTag

HaloTag is a self-labeling protein tagging system based on a mutant bacterial haloalkane dehalogenase enzyme.[1] It can be genetically fused to a protein of interest to label it for fluorescence imaging experiments. The HaloTag protein binds irreversibly to special chloroalkane ligands. For imaging, fluorescent ligands are used. These ligands bind irreversibly to the HaloTag protein. The resulting complex is fluorescent and may be used to image the protein of interest.



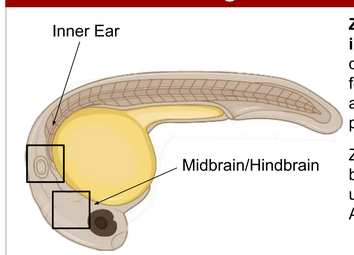
HaloTag has multiple advantages which make it a good candidate for imaging in zebrafish.

1. HaloTag has many different fluorescent ligands available. Each ligand has a different excitation and emission wavelength.
2. The timing of fluorescence can be controlled. HaloTag only fluoresces when bound to the ligand, so timing of fluorescence and tagging can be precisely controlled.
3. The tunability of the HaloTag system means there are great opportunities for multiplexing. By slightly modifying the HaloTag protein, the same ligand can result in different fluorescence lifetimes and be used for multiplexing of a single channel.[2]
4. Different proteins can be used as the protein of interest, as HaloTag isn't specific to a given protein.



Structure from Kang *et al. Chemical Communications.* 2017.

Background: Imaging Zebrafish

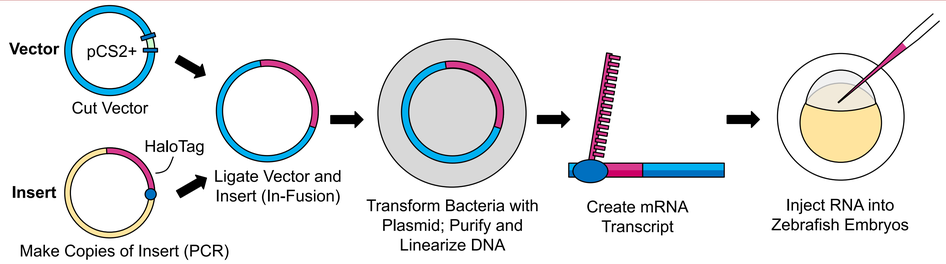


Created with BioRender.com.

Zebrafish are ideal model organisms for imaging in developmental biology studies due to their transparency and external fertilization. The transparency of the embryos allows for noninvasive imaging of biological processes using fluorescence microscopy.[3]

Zebrafish also have great similarity to human brain organization and could therefore be useful for studying diseases such as Alzheimer's Disease.[4]

Materials and Methods



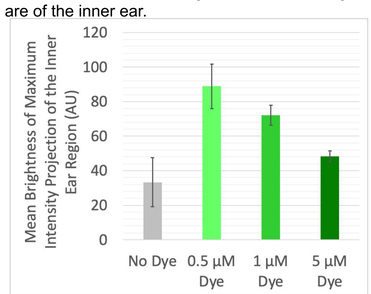
To test the effectiveness of HaloTag in zebrafish embryos, an expression construct expressing HaloTag was created. The pCS2+ vector was used expression of HaloTag. The plasmid was cloned in bacteria, purified, and used to create an mRNA transcript for injection into single-cell zebrafish embryos. 100 pg of mRNA was injected into each zebrafish embryo.

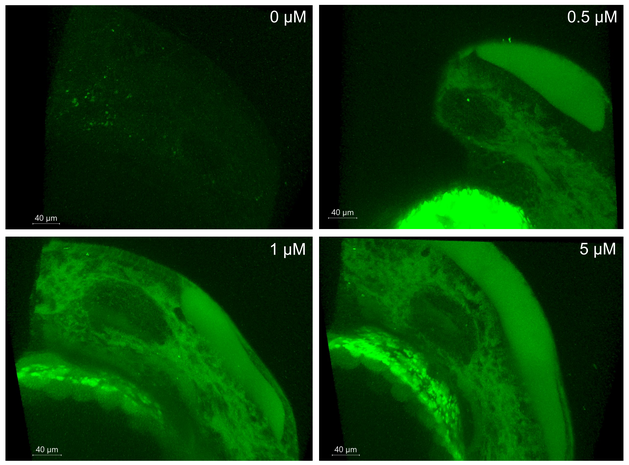
Zebrafish were imaged at about 28hpf on an inverted confocal microscope (Zeiss LSM 880). Zebrafish with dye were incubated in embryo media (60mg/ml of Instant Ocean and 75mg/ml of $CaSO_4$ in ddH_2O) with a certain concentration of Oregon Green 488 dye (the fluorescent ligand) and washed before imaging.

Results: Determination of Ideal Dye Concentration for HaloTag

Before imaging zebrafish with HaloTag, wildtype zebrafish were incubated with different concentrations of dye to determine nonspecific binding of the ligand in the absence of HaloTag.

There is no significant difference between 1 µM dye and other concentrations; 1 µM dye was used for the HaloTag tests. These images are of the inner ear.

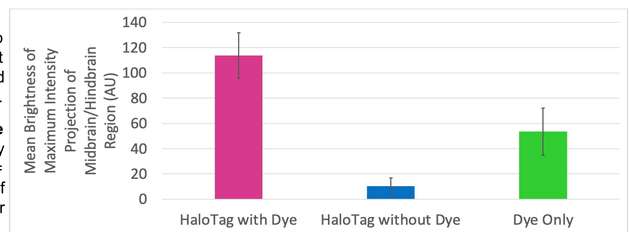


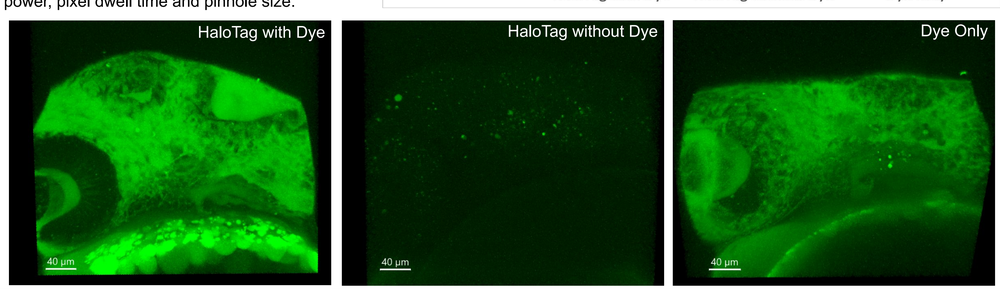


Results: HaloTag with Ligand

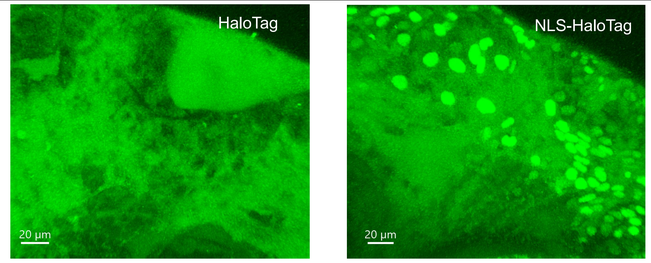
HaloTag was tested in zebrafish. The HaloTag with dye images were compared to HaloTag alone (to demonstrate that HaloTag without the dye does not fluoresce) and dye alone (to identify the increased fluorescence from binding to HaloTag).

HaloTag with dye is twice as bright than dye alone $t(3) = 4.63$, $p = 0.0095$. It is also significantly brighter than HaloTag without dye $t(4) = 10.16$, $p = 0.0003$. Images of the midbrain/hindbrain region of the zebrafish embryo taken with the same laser power, pixel dwell time and pinhole size.



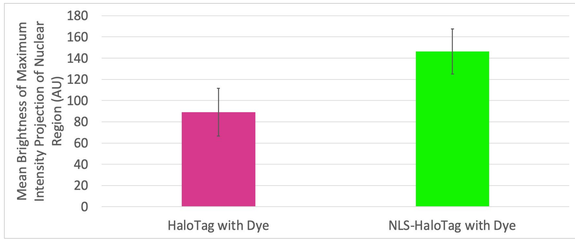


Results: Nuclear Localization Signal (NLS) HaloTag



In addition to testing HaloTag without a localization signal, which should be expressed throughout the cell, NLS-HaloTag was also tested. NLS-HaloTag localize primarily within the nucleus. The mRNA was provided by Falk Schneider.

NLS-HaloTag is twice as bright than regular HaloTag in the nuclei $t(5) = 3.71$, $p = 0.0069$. Images of the midbrain/hindbrain regions taken with the same laser power and dwell.



Summary and Future Research

HaloTag was demonstrated to be effective in zebrafish embryos, being brighter than dye alone and HaloTag without dye. HaloTag with dye is almost twice as bright as dye alone. NLS-HaloTag is significantly brighter than regular HaloTag, particularly in the nuclei. Concentration of Oregon Green dye does not appear to have a significant effect on the brightness of images without HaloTag.

There are two primary paths of future research. Refining the dying process is necessary to reduce nonspecific binding of the fluorescent ligand without HaloTag. A genetic fusion of HaloTag and a protein such as amyloid beta or tau proteins could demonstrate HaloTag's effectiveness with an actual protein of interest and increase understanding of the protein clearance issues involved in Alzheimer's Disease.

Acknowledgments

Le Trinh provided the great opportunity to work on this project. Emily Shaw mentored me throughout the summer. Thanks to Falk Schneider for the basis of this project and the NLS-HaloTag mRNA and Arkadi Schwartz for instructing me on the use of the microscope. The Fraser Lab and Bridge Institute provided other support and a stipend. Medha Singamsetty helped with me throughout both of our projects, especially on the molecular work.

References

[1] Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol* **3,** 373-82 (2008). https://doi.org/10.1021/cb800025k.

[2] Frei, M.S., Tarnawski, M., Roberti, M.J. et al. Engineered HaloTag variants for fluorescence lifetime multiplexing. *Nat Methods* **19**, 65–70 (2022). https://doi.org/10.1038/s41592-021-01341-x

[3] Choi, TY., Choi, TI., Lee, YR. *et al.* Zebrafish as an animal model for biomedical research. *Exp Mol Med* **53**, 310–317 (2021). https://doi.org/10.1038/s12276-021-00571-5

[4] Saleem, S., Kannan, R.R. Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospecific drug discovery. *Cell Death Discov.* **4**, 45 (2018). https://doi.org/10.1038/s41420-018-0109-7

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