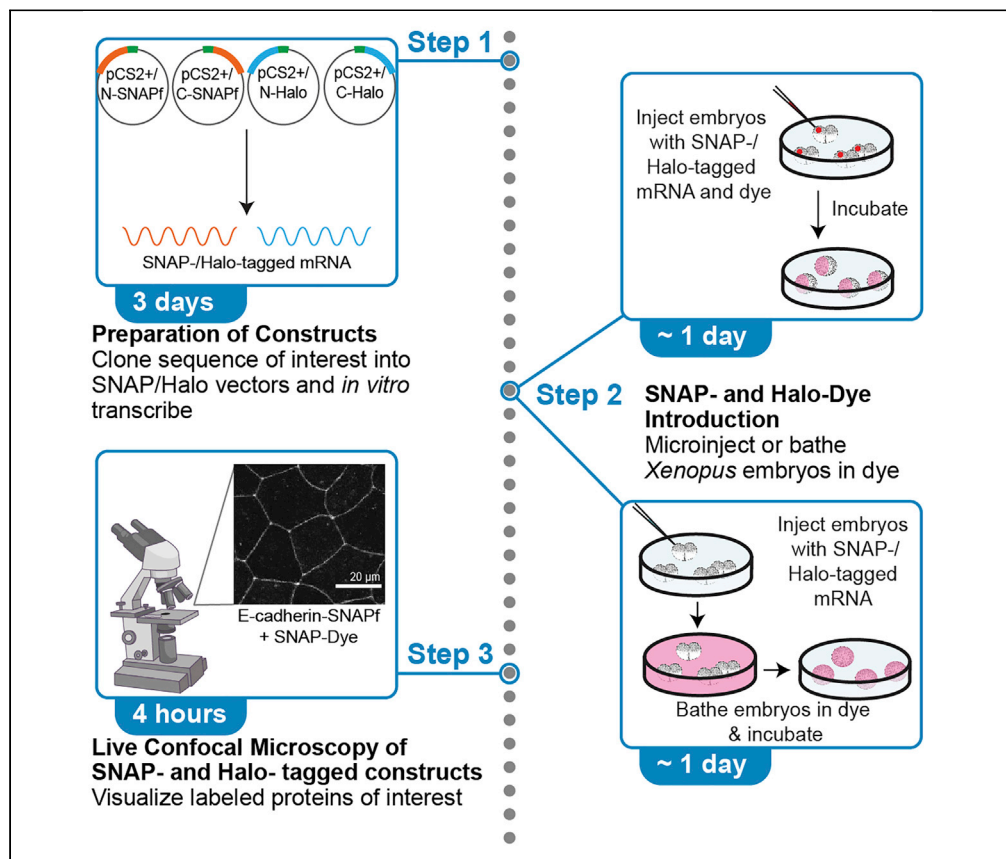


Protocol

SNAP- and Halo-tagging and dye introduction protocol for live microscopy in *Xenopus* embryos



Traditional fluorescent proteins exhibit limitations in brightness and photostability that hinder optimal characterization of the dynamic cellular behavior of proteins of interest. SNAP- and Halo-tagging are alternatives to traditional fluorescent protein tagging utilizing bright, stable chemical dyes, which may improve signal-to-noise ratio. However, there has been limited use of this approach *in vivo* in developing organisms. Here, we present a protocol for implementing SNAP- and Halo-tagging in gastrula-stage *Xenopus laevis* embryos for live confocal microscopy.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Preparing SNAP- and Halo-tagged pCS2+-based constructs

Microinjecting SNAP- and Halo-tagged mRNAs into *Xenopus* embryos

Introducing SNAP- and Halo-dyes in developing *Xenopus* embryos

Visualizing SNAP- and Halo-tagged proteins with live confocal microscopy

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Protocol

SNAP- and Halo-tagging and dye introduction protocol for live microscopy in *Xenopus* embryosClaire E. Dudley,^{1,3,*} Lotte van den Goor,¹ and Ann L. Miller^{1,2,4,*}¹Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1085, USA²Cellular and Molecular Biology Graduate Program, University of Michigan, Ann Arbor, MI 48109-1085, USA³Technical contact⁴Lead contact*Correspondence: dudleyc@umich.edu (C.E.D.), annlm@umich.edu (A.L.M.)
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SUMMARY

Traditional fluorescent proteins exhibit limitations in brightness and photostability that hinder optimal characterization of the dynamic cellular behavior of proteins of interest. SNAP- and Halo-tagging are alternatives to traditional fluorescent protein tagging utilizing bright, stable chemical dyes, which may improve signal-to-noise ratio. However, there has been limited use of this approach *in vivo* in developing organisms. Here, we present a protocol for implementing SNAP- and Halo-tagging in gastrula-stage *Xenopus laevis* embryos for live confocal microscopy.

For complete details on the use and execution of this protocol, please refer to Varadarajan et al. (2022).

BEFORE YOU BEGIN

In this protocol, we introduce pCS2+-based DNA vectors for tagging a sequence of interest with a SNAP- or Halo-tag, present different methods for dye introduction, and provide examples of live confocal imaging of SNAP- or Halo-tagged proteins with different dyes in gastrula-stage *Xenopus laevis* embryos. In the present case, we focus on labeling cell-cell junction proteins including adherens junction and tight junction proteins of interest. Essential materials necessary for this protocol include *Xenopus* embryos, a confocal microscope, constructs encoding SNAP- and Halo-tagged proteins of interest, materials for *in vitro* transcription, and SNAP- and Halo-dyes. SNAP- and Halo-tagged constructs must be cloned or acquired for the desired proteins of interest. Empty versions of the SNAP- and Halo- pCS2+ vectors we generated for this protocol are available through Addgene. Appropriate SNAP- and Halo-constructs must be *in vitro* transcribed from linearized pCS2+ plasmids, and resulting mRNAs are microinjected into 2–4 cell stage *Xenopus* embryos.

Institutional permissions

All studies conducted using *Xenopus laevis* embryos strictly adhered to the compliance standards of the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the University of Michigan Institutional Animal Care and Use Committee. Please note that readers will need to acquire approval to work with vertebrate animals at their relevant institutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NotI-HF	NEB	Cat #: R3189
KpnI-HF	NEB	Cat #: R3142

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SP6 mMessage mMachine	Ambion	Cat #: AM310
RNeasy MinElute Clean-up Kit	QIAGEN	Cat #: 74204
L-Cysteine	Sigma	Cat #: 168149
Human Chorionic Gonadotropin (HCG)	Fisher	Cat #: ICN19859110
SNAP-Cell 647-SiR	NEB	Cat #: S9102S
SNAP-Cell TMR-Star	NEB	Cat #: S9105S
SNAP-Cell Oregon Green	NEB	Cat #: S9104S
Janelia Fluor 646 HaloTag (No-Wash)	Promega	Cat #: GA1120
Janelia Fluor 549 HaloTag (No-Wash)	Promega	Cat #: GA1110
Oregon Green HaloTag	Promega	Cat #: G2802
Experimental models: Organisms/strains		
Oocyte Positive Female <i>Xenopus laevis</i> , Pigmented or Albino, 10.5–11 cm	Xenopus 1	Cat #: 4280
Male (Mature) <i>Xenopus laevis</i> , Pigmented	Xenopus 1	Cat #: 4290
Recombinant dNA		
pCS2+/N-SNAPf	This study	Addgene 184415
pCS2+/C-SNAPf	This study	Addgene 184416
pCS2+/N-Halo	Varadarajan et al. (2022)	Addgene 184417
pCS2+/C-Halo	This study	Addgene 184418
pCS2+/E-cadherin-SNAPf	This study	
pCS2+/SNAPf-Vinculin	This study	
pCS2+/Halo-ZO-1	This study	
pCS2+/Halo-Vinculin	This study	
pCS2+/Vinculin-3xGFP	Higashi et al. (2016)	
pCS2+/Vinculin-GFP	This study	
pCS2+/mNeon-Vinculin	This study	
pCS2+/Vinculin-mNeon	Arnold et al. (2019)	
Software and algorithms		
FIJI-Image J	https://imagej.net/software/fiji/downloads	
Other		
35 mm × 10 mm Petri Dishes	Fisher	Cat #: FB0875711YZ
60 mm × 15 mm Petri Dishes	Fisher	Cat #: FB0875713A
96 Well Polypropylene Storage Microplate	Fisher	Cat #: AB-0796
Transfer Pipets	Fisher	Cat #: 137119D
Microinjector (BTX MicroJect 1000A)	Harvard Apparatus	Cat #: 45-0750
Micromanipulator for Glass Needle	NARISHIGE	Cat #: MN-153
Mineral Oil	Sigma	Cat #: M5904-5X5ML
10 μL Drummond Glass Capillaries	Fisher	Cat #: 21-169A
P-80/PC Flaming Brown Micropipette Puller	Sutter Instrument	
Fluoview 1000 Microscope and FV10-ASW Software	Olympus	

MATERIALS AND EQUIPMENT

1 × MMR

Reagent	Final concentration	Amount
NaCl	100 mM	58.44 mg
KCl	2 mM	1.49 mg
CaCl ₂	2 mM	2.22 mg
MgCl ₂	1 mM	0.95 mg
HEPES	5 mM	11.92 mg
Milli-Q water	N/A	1 L
Total	N/A	1 L

pH adjusted to 7.4 using 1 M NaOH. Stable at room temperature (22°C) for several months.

2% Cysteine

Reagent	Final concentration	Amount
L-Cysteine	2%	2 g
1 × MMR	N/A	100 mL
Total	N/A	100 mL

pH adjusted to 7.8 using 10 M NaOH. Stable at room temperature (22°C) for 1 day.

⚠ **CRITICAL:** Human Chorionic Gonadotropin (HCG) is a reproductive hazard. Reproductive hazards may affect the reproductive health of women or men. Potential health effects include infertility, miscarriage, birth defects, and developmental disorders in children. Caution should be taken in both preparation and disposal of HCG. Minimum PPE for handling during preparation and injection of HCG is wearing a lab coat, eye protection, and gloves.

Alternatives: N/A.

STEP-BY-STEP METHOD DETAILS

Part 1: Preparation of SNAP- and Halo-tagged constructs for expressing SNAP/Halo-tagged proteins of interest in *Xenopus* embryos

⌚ **Timing:** 3 days

This step prepares the materials (SNAP/Halo-tagged DNA constructs, mRNAs, fertilized *Xenopus* embryos) necessary for live imaging of SNAP/Halo-tagged proteins of interest in live *Xenopus* embryos. The frog handling portion of the protocol is adapted from [Sive et al. \(2010\)](#).

⚠ **CRITICAL:** To successfully visualize your SNAP- or Halo-tagged protein of interest, it is imperative that the sequence of interest is cloned into the SNAP- or Halo-expression vector in frame without intervening stop codons.

Note: SNAPf is an updated version of SNAP-tag, that is optimized for faster substrate labeling ([Cole, 2013](#)).

Note: To optimize expression of the SNAP- and Halo-tagged proteins in *Xenopus* embryos, we codon optimized both tags: SNAPf (codon 436), Halo (codons 82, 121, 154, 166, 403, 511, 517, 535, 571, 643, 655, 700, 742, 802, 832, 856, 868, 871).

Note: Both SNAP- and Halo-tagging systems appear to be equally effective in tagging and visualizing proteins of interest. Differences in visualization between SNAP- and Halo-tagging appear to be a result of dye differences, rather than the tag.

- Clone sequence of interest into the appropriate SNAPf/Halo pCS2+ vector backbone ([Figure 1](#)).
 - Clone sequence of interest into the multiple cloning site (MCS) that is directly upstream or downstream of the SNAPf- or Halo-tag.

⏸ **Pause point:** The use of these constructs is not time sensitive, DNA can be stored at –20°C for later use.

- In vitro* transcribe the plasmid containing the SNAPf-/Halo-tagged sequence of interest.
 - Linearize the vector using NotI or KpnI restriction enzymes.

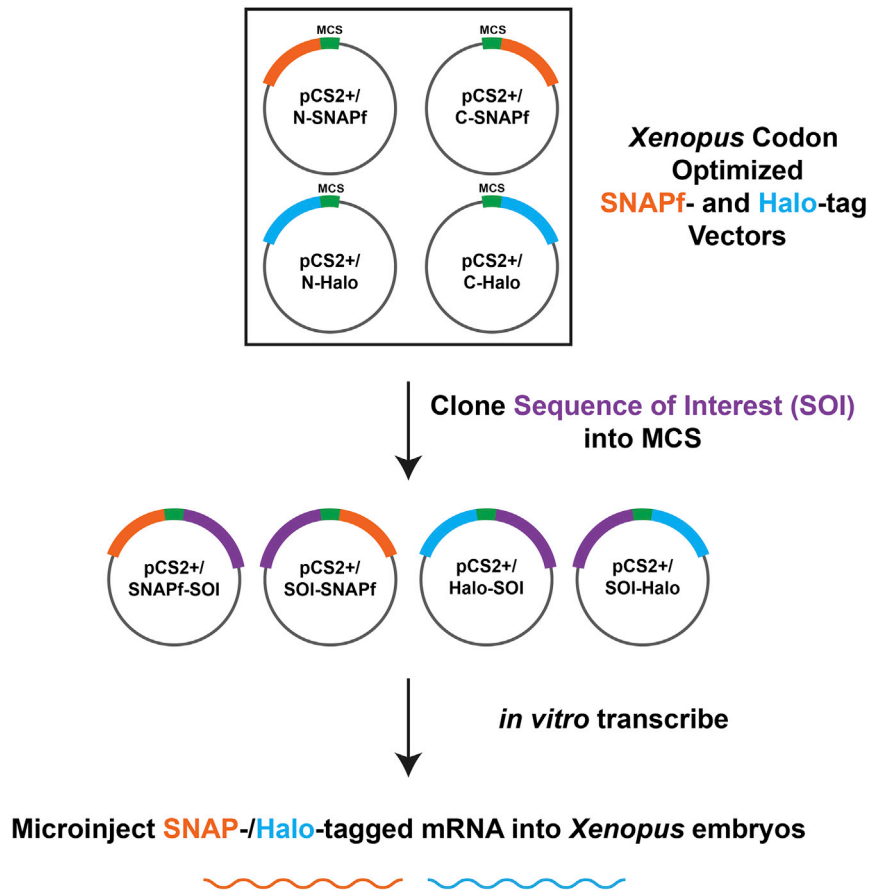


Figure 1. Workflow for expression of pCS2+/SNAPf- and Halo-tagged constructs in *Xenopus* embryos

pCS2+/SNAPf- and Halo-vectors that were codon optimized for *Xenopus* can be acquired from Addgene. The sequence of interest is cloned into the multiple cloning site (MCS), located upstream/downstream of the SNAPf/Halo-tag. Tagged constructs are *in vitro* transcribed to generate mRNA that is microinjected into 2–4 cell stage *Xenopus* embryos.

Note: Make sure the sequence of interest does not contain the restriction site for the restriction enzyme used.

Note: To check for proper linearization, the vector should be run on a gel before proceeding to *in vitro* transcription.

- b. *In vitro* transcribe the linearized plasmid following the instructions in the SP6 mMessage mMachine kit.
- c. Purify the mRNA following the instructions in the RNeasy kit.

Note: The mRNA should be run on a gel before experimental use to ensure expected size and purity.

▣ **Pause point:** The use of these mRNAs is not time sensitive, mRNA can be stored at -80°C for later experiments.

3. Prime the adult female *Xenopus laevis* frog by injecting 50 units of human chorionic gonadotropin (HCG) into the dorsal lymph sac several days before the experiment (e.g., 3–7 days).
4. The afternoon before egg collection (e.g., 14–24 h before egg collection), induce ovulation by injecting 400–800 units of HCG into the dorsal lymph sac of the primed frog.

5. Collect eggs from the adult female frog (Sive et al., 2010) in a petri dish filled with 1× MMR.
 - a. Using a transfer pipette, remove most of the MMR, leaving ~1/10 the of the 1× MMR remaining.
6. Fertilize the eggs with about one quarter of a testes harvested from an adult male *Xenopus laevis* frog (Sive et al., 2010).
 - a. Macerate the testes using forceps to ensure release of the sperm.
 - b. Wait 1 min, and fill the dish with water, resulting in ~0.1× MMR.
7. Dejelly the embryos at least 30 min post-fertilization with 2% Cysteine (in 1× MMR) adjusted to pH 7.8 (Sive et al., 2010).
 - a. Replace media on embryos with cysteine solution, transfer embryos to a 50 mL falcon tube, bring volume of cysteine solution to ~25 mL.
 - b. Gently swirl the embryos. Once the embryos lose their jelly coating and settle to the bottom of the falcon tube, pour out the cysteine.
 - c. Rinse the embryos twice with 1× MMR and twice with 0.1× MMR.
8. Separate the embryos into petri dishes, removing any unhealthy embryos. Incubate the embryos in 0.1× MMR until they reach 2–4 cell stage (~1.5–2 h post-fertilization).
 - a. Incubate dishes of embryos at 13, 15, 17°C, and room temperature (RT, 22°C) to spread the window of time embryos are at 2–4 cell stage to allow more time for microinjection.

Note: This range of temperatures supports normal development of *Xenopus* embryos.

Part 2: SNAP- and Halo-dye introduction

⌚ Timing: 18 h – 1 day

This step provides two options for introducing SNAP- and Halo-dyes to intact *Xenopus* embryos.

Note: There are several possible methods for dye introduction (other potential methods discussed in [troubleshooting](#)). Co-microinjecting the dye with mRNAs or bathing the embryos in dye are the most straightforward dye introduction approaches for intact *Xenopus* embryos (Figure 2) (see Problem 4 in [troubleshooting](#)). Both methods are compatible with the expression of other conventional fluorescently-tagged proteins.

Note: Optimization of both dye concentration and amount of microinjected mRNA is necessary to achieve optimal visualization (see Problem 1 in [troubleshooting](#)).

Note: In our experience, Halo-tag paired with a “no-wash” Janelia Fluor dye led to optimal visualization.

Note: Timing depends on the temperature the embryos are incubated at (e.g., 13, 15, 17°C, and RT (22°C)) and the developmental stage that you want to image, and can be modified to align with your scheduled imaging time.

Co-microinjecting dye with mRNA

Before microinjecting, prepare materials for microinjection:

- i. Prepare microinjection needles. Pull needles using 10 µL glass capillary tubes to create needles with a long, thin point.
- ii. Set-up microinjector, turn on N₂ tank.
- iii. Calibrate the needle. Break the end of the needle using forceps. Attach needle to microinjector tubing. Fill the needle with RNase free water, and adjust the pressure and injection time on the microinjector so that it produces a 5 nL droplet in mineral oil.

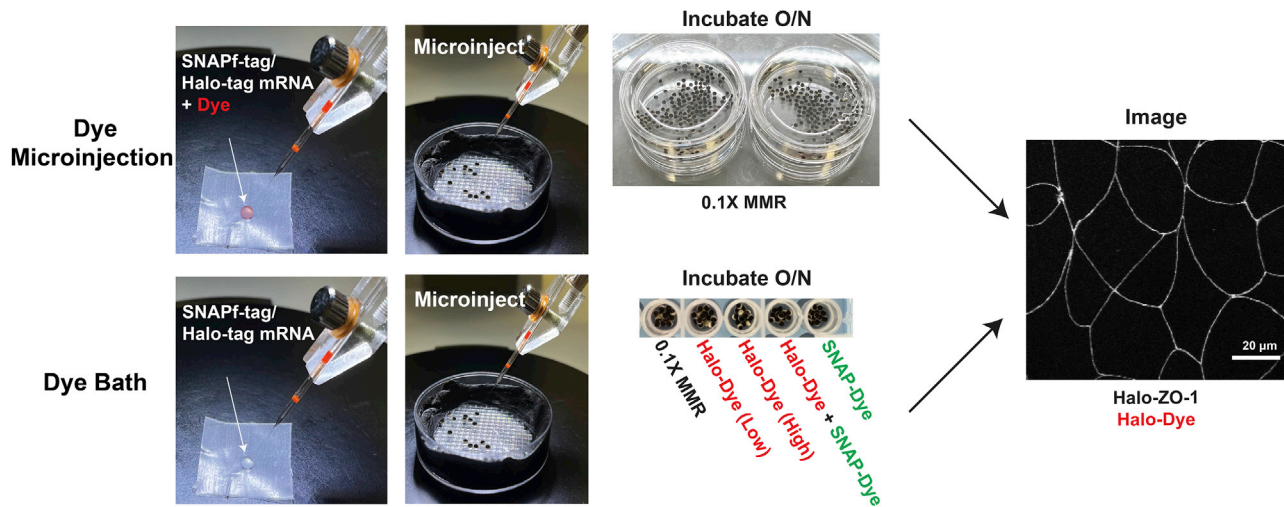


Figure 2. Dye introduction methods for labeling SNAPf- or Halo-tagged proteins expressed in intact *Xenopus* embryos

(Top) SNAP/Halo dyes can be microinjected with SNAPf- or Halo-tagged mRNAs. (Bottom) After microinjecting SNAPf- or Halo-tagged mRNAs, embryos can also be bathed in SNAP/Halo dyes overnight (O/N, 15–22 h) for next-day imaging.

9. Resuspend SNAP- or Halo-dye aliquot to an appropriate stock concentration using RNase free water.

△ **CRITICAL:** Vortex to ensure even suspension of the dye.

Note: Precautions to maintain dye integrity should be made by working with the dyes in minimal light during resuspension and incubation. Any overhead lights in the room should be turned off during dye resuspension, to reduce the risk of dye breakdown. During incubation, embryos are kept in dark incubators with tinted windows.

Note: Stock concentration of dye aliquots used in the SNAP- and Halo-experiments in this protocol was 200 µM. We recommend keeping aliquots at least 10-fold higher than the concentration of dye used in experiments (see Problem 5 in [troubleshooting](#)).

10. Mix the dye and mRNA encoding the proteins of interest to make a microinjection mix.
 - a. Mix the microinjection droplet by pipetting up and down with a micropipetter to ensure an even distribution of the dye and mRNA within the droplet (the droplet may exhibit a color depending on the concentration of dye(s) in the droplet).
 - b. Fill the needle with the microinjection mix.

Note: SNAP- and Halo-dyes can be co-microinjected in the same microinjection mix.

Note: We dilute dye and mRNA to a 5× stock concentration and then add 1 µL of each to a droplet that has a final volume of 5 µL. If needed, RNase free water is added to raise the droplet volume to 5 µL.

Note: The concentration of microinjected SNAP- or Halo-tagged mRNA was generally the same concentration used for the equivalent mNeon-tagged mRNA for the protein of interest but would need to be determined empirically.

11. Inject 2–4 cell stage *Xenopus* embryos with 5 nL of the microinjection mix at two distinct locations within the animal hemisphere.

Note: To target epithelial cells, we injected in the animal hemisphere. However, injections could be targeted to any region of interest according to the *Xenopus* fate map.

Note: We injected embryos in 0.1× MMR. Injections can also be performed in 5% Ficoll (in 0.1× MMR).

12. Incubate the embryos co-microinjected with dye and mRNA in 35 mm × 10 mm petri dishes filled with 0.1× MMR, leaving enough space so that the embryos are not crowded.
 - a. Incubate the embryos in a dark incubator at 15°C or 17°C overnight (15–22 h) for next-day imaging of embryos at gastrula stage.

Bathing embryos in dye

Before bathing embryos in dye, prepare materials for microinjection, and microinject the embryos:

- i. Prepare microinjection needles. Pull needles using 10 µL glass capillary tubes to create needles with a long, thin point.
 - ii. Set-up microinjector, turn on N₂ tank.
 - iii. Calibrate the needle. Break the end of the needle using forceps. Attach needle to microinjector tubing. Fill the needle with RNase free water, and adjust the pressure and injection time on the microinjector so that it produces a 5 nL droplet in mineral oil.
13. Prepare the 5 µL microinjection mix (see step 10, but don't add dye), containing mRNA encoding the proteins of interest, and fill the needle with the microinjection mix.

Note: The concentration of microinjected SNAP- or Halo-tagged mRNA was generally the same concentration used for the equivalent mNeon-tagged mRNA for the protein of interest but would need to be determined empirically.

14. Inject 2–4 cell stage *Xenopus* embryos with 5 nL of the microinjection mix at two distinct locations within the animal hemisphere.
15. Incubate the embryos microinjected with mRNA in petri dishes filled with 0.1× MMR at 17°C until dye bath is prepared in the 96-well plate.
16. Resuspend SNAP- or Halo-dye aliquot to working concentration using 0.1× MMR, and add 300 µL of the resuspended dye to a single well within a 96-well plate.

△ **CRITICAL:** Pipette thoroughly to ensure a homogeneous dye solution.

17. Using a transfer pipette, add 10–15 *Xenopus* embryos microinjected with SNAP and/or Halo mRNAs to the well.

△ **CRITICAL:** When adding the embryos to the well, touch the tip of the micropipette to the surface of the dye bath, allowing the embryos to drop into to the well, without transferring additional 0.1× MMR.

Note: No more than 15 embryos were added to each well to facilitate dye diffusion.

Note: SNAP- and Halo-dyes can be included in the same dye bath (Figure 2).

18. Incubate the 96-well plate at 15 or 17°C overnight (15–22 h) for next-day imaging of embryos at gastrula stage.

Part 3: Live confocal imaging of SNAP- and Halo-tagged proteins

⌚ **Timing:** 4 h

This step allows you to visualize the SNAP- and/or Halo-tagged protein of interest bound to its dye partner in gastrula-stage *Xenopus* embryos using confocal microscopy.

19. Remove gastrula-stage (Nieuwkoop and Faber stage 10.5–12) *Xenopus* embryos from the incubator.
 - a. Using a dissecting microscope, select the embryos that will be prepared for live imaging.

△ **CRITICAL:** Choose embryos that appear healthy and have even pigmentation.

Note: For embryos bathed in dye, transfer the embryos from the 96-well plate to a petri dish containing 0.1 × MMR before viewing.

20. Prepare imaging slide (Woolner et al., 2009).

Note: There are multiple methods that can be used to mount *Xenopus* embryos for live imaging. We use a 0.8 mm thick custom metal slide that has a 5 mm circular hole cut in the center.

- a. Apply a thin layer of vacuum grease to both sides of the slide.
 - b. Place a square coverslip on one side of the slide, making a well.
 - c. Add 3 embryos and a small volume of 0.1 × MMR to the well.
 - d. Place a second square coverslip on top of the embryos, gently sandwiching the embryos between the two glass coverslips.
21. Using a confocal microscope (we use a 60× objective on an inverted Olympus FV1000), focus on one of the embryos.
 22. Imaging can now be conducted as it would for any other fluorescently-labeled protein of interest expressed in *Xenopus* embryos.

Note: Some dyes, including the Janelia Fluors, possess a very bright signal, so laser power may need to be adjusted to very low levels.

Note: Several factors contribute to successful visualization of SNAP- and Halo-tagged proteins (see Problems 1, 2, and 3 in [troubleshooting](#)).

Note: We used FIJI software to process the images presented in this protocol.

EXPECTED OUTCOMES

Use of SNAP- and Halo-tags has been well-documented in cell culture, with limited application in the *Xenopus* model system (Kuriyama et al., 2014; Ollech et al., 2020; Varadarajan et al., 2022). In this protocol, we introduce pCS2+-based SNAP- and Halo-tag constructs we developed, which are optimized for use in *Xenopus* embryos and allow for tagging on the N- or C-terminus of a protein of interest. Additionally, we present two SNAP/Halo dye introduction methods that are compatible with intact *Xenopus* embryos expressing SNAP- and Halo-tagged proteins. The microscopy images visualizing SNAP- and/or Halo-labeled proteins of interest accompanying this protocol were captured with a scanning confocal microscope, but other types of microscopy could also potentially be used.

Commercially-available as well as freely-available (Lavis, 2021) SNAP- and Halo-dyes are offered across the fluorescence spectrum and are compatible with conventional microscopy laser lines (e.g., 488, 559, 635 nm). We show here that various dyes of different wavelengths can be used to visualize SNAP- or Halo-tagged proteins of interest in developing wild type (WT, pigmented) and albino *Xenopus* embryos (Figure 3) through either co-microinjecting dye or bathing embryos in dye.

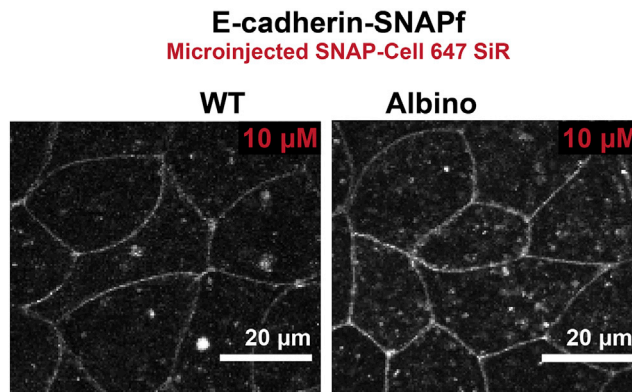


Figure 3. SNAP-tagged proteins can be visualized in both wild type (WT) and albino gastrula-stage *Xenopus laevis* embryos

Images of WT (left) and Albino (right) *Xenopus laevis* embryos co-microinjected with E-cadherin-SNAPf mRNA (50 pg) and SNAP-Cell 647 SiR dye (10 μ M). Scale Bars: 20 μ m.

Note: Both dye microinjection and dye baths are compatible methods for SNAP- and Halo-tagging in developing *Xenopus* embryos. To achieve optimal visualization using this system, we recommend first trying dye microinjection along with a “wash-free” dye. However, dye baths may be more fitting for certain experimental conditions.

SNAP- and Halo-tagging can be used to visualize proteins of interest at various subcellular locations. In this protocol, we focus on SNAP- and Halo-tagging of E-cadherin (Figure 4) and ZO-1 (Figure 5), which are found in epithelial cells at adherens junctions and tight junctions, respectively. We also successfully visualized Vinculin using either a SNAP- or Halo-tag (Figure 6). Vinculin, which is recruited to adherens junctions under high tension (Yonemura et al., 2010; Higashi et al., 2016), localizes characteristically in three distinct puncta at tricellular junctions (Higashi and Miller, 2017), but Vinculin can be challenging to image to due to weak signal and variable tissue tension conditions in live embryos. The ability to use bright chemical dyes is a useful strategy to improve the signal-to-noise ratio for hard to image proteins like Vinculin. We found that smaller, brighter fluorophores, like mNeon and Halo-tag + Halo-dye, are better able to visualize Vinculin’s characteristic three puncta localization at tricellular junctions (Figure 7). The establishment of SNAP- and Halo-tagged Vinculin also expanded our ability to visualize Vinculin to new wavelengths by using red or far-red dyes, which will be useful for future experimental directions. Together, these examples highlight how SNAP- and Halo-tagging can be used to visualize junctional proteins in *Xenopus laevis* embryos.

Note: In the study by Varadarajan et al., 2022, our lab used Halo-tagged PKC β -C2 domain to probe for calcium in *Xenopus* embryos, demonstrating that a cytoplasmic Halo-tagged protein can also be visualized in *Xenopus* embryos.

A key advantage of SNAP- and Halo-tags is the experimental flexibility. Since SNAP/Halo-tagged proteins are not genetically labeled by a single fluorescent protein, there is a greater degree of experimental flexibility. For example, different dyes can be used to label the same construct depending on the desired experimental parameters (see Figures 4–6 and 8). A SNAP-tagged protein and a Halo-tagged protein can also be simultaneously expressed and labeled with appropriate SNAP- and Halo-dyes through either co-microinjecting dyes or bathing embryos in both dyes (Figures 2 and 8).

Far-red dyes, which are desirable for some super-resolution microscopy approaches, are available for both SNAP- and Halo-tags. The far-red dyes were also of particular interest to us because they

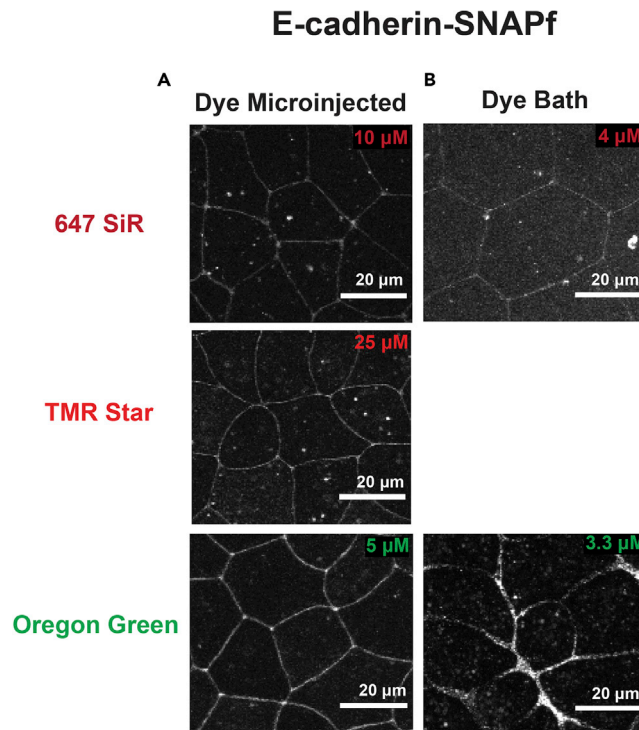


Figure 4. Microinjecting or bathing embryos in dye can label E-cadherin-SNAPf *in vivo*

(A) Images of *Xenopus* embryos expressing E-cadherin-SNAPf (50 pg) labeled via dye microinjection with SNAP-Cell 647 SiR (10 μ M), SNAP-Cell TMR Star (25 μ M), or SNAP-Cell Oregon Green (5 μ M).

(B) Images of *Xenopus* embryos expressing E-cadherin-SNAPf (50 pg) labeled via dye bath with SNAP-Cell 647 SiR (4 μ M) or SNAP-Cell Oregon Green (3.3 μ M).

Scale Bars: 20 μ m.

allow for visualization of an additional protein of interest when other proteins are already tagged with mNeon/GFP or mCherry or when experimental constraints (e.g., optogenetic systems) are already utilizing the green and red wavelengths (Varadarajan et al., 2022; Yamamoto et al., 2021).

Note: We have tried several far-red fluorescent proteins (e.g., mPlum, mKate2, TagRFP657, and iRFP) in *Xenopus* without much success; recently, we tried miRFP703 with success (Yamamoto et al., 2021).

LIMITATIONS

Optimization of dye concentration is necessary for labeling different proteins of interest

This protocol recommends two different methods for dye introduction, with some guidance for dye concentrations tested for several SNAP- or Halo-tagged junctional proteins. When we were first optimizing this system for use in *Xenopus* embryos, we focused on E-cadherin and ZO-1 because they are consistently easily visualized junctional proteins. After initial optimization of dye concentrations and dye introduction approaches, we tested another junctional protein, Vinculin, whose junctional localization was more variable, necessitating additional dye optimization. The dye concentrations suggested here should be considered as starting points to empirically determine the right dye concentration for your application.

Some dyes are more compatible with the *Xenopus* embryo model system than others

Traditional SNAP- and Halo-dye introduction protocols for cell culture call for several washes after dye incubation in order to remove excess dye, which could cause background noise when imaging (Cole, 2013; Erdmann et al., 2019; Grimm et al., 2015; Los et al., 2008). However, *Xenopus laevis*

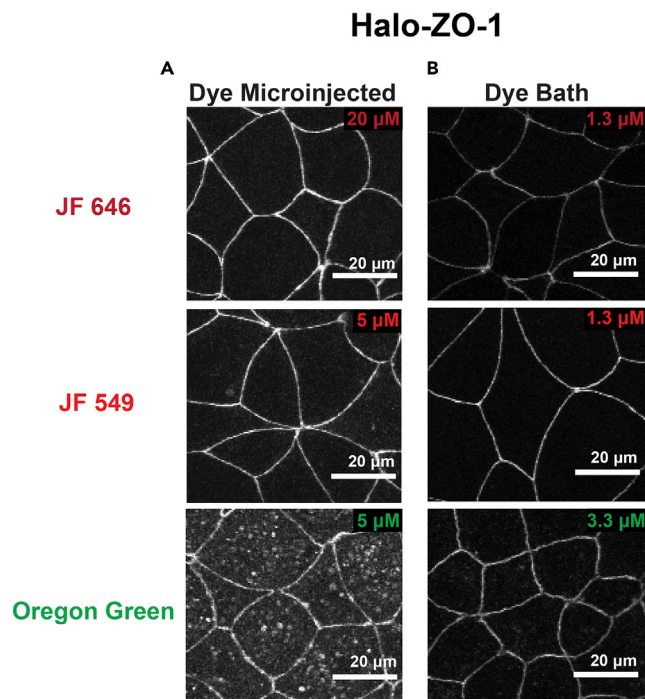


Figure 5. Microinjecting or bathing embryos in dye can label Halo-ZO-1 in vivo

(A) Images of *Xenopus* embryos expressing Halo-ZO-1 (100 pg) labeled via dye microinjection with Janelia Fluor 646 HaloTag (20 μ M), Janelia Fluor 549 HaloTag (5 μ M), or Oregon Green HaloTag (5 μ M).

(B) Images of *Xenopus* embryos expressing Halo-ZO-1 (100 pg) labeled via dye bath with Janelia Fluor 646 HaloTag (1.3 μ M), Janelia Fluor 549 HaloTag (1.3 μ M), and Oregon Green HaloTag (3.3 μ M).

Scale Bars: 20 μ m.

embryos are quite large (1.2 mm in diameter) and are encased by a vitelline envelope, a semipermeable membrane that regulates nutrient diffusion and provides structural support. Washes are therefore not an effective method for removing excess dye, which can lead to higher background signal in *Xenopus* embryos. Thankfully, there are “no-wash” dyes that are designed to eliminate the need for washing away excess dye; however, the wash free characteristic is limited to a small portion of free and commercially-available dyes. In our hands, the “no-wash” dyes tend to work well in *Xenopus* embryos. Alternatively, the vitelline could be removed manually or enzymatically to improve dye and wash diffusion within the embryo; however, removing the vitelline is challenging, time consuming, and results in very delicate embryos, so it is not an ideal solution. In summary, testing different dyes is essential to harnessing the full potential of this tagging system, and some dyes may be better suited for certain experimental conditions than others.

Healthy embryos are essential for clean microscopy images

Poor embryo health can impede visualization of SNAP/Halo-labeled proteins of interest. Unhealthy embryos may not express the SNAP- or Halo-constructs optimally. Additionally, we find that unhealthy embryos tend to form dye aggregates that increase background noise.

TROUBLESHOOTING

Problem 1

Signal is not bright enough.

Potential solution

- Embryos are unhealthy.

Dye Microinjected

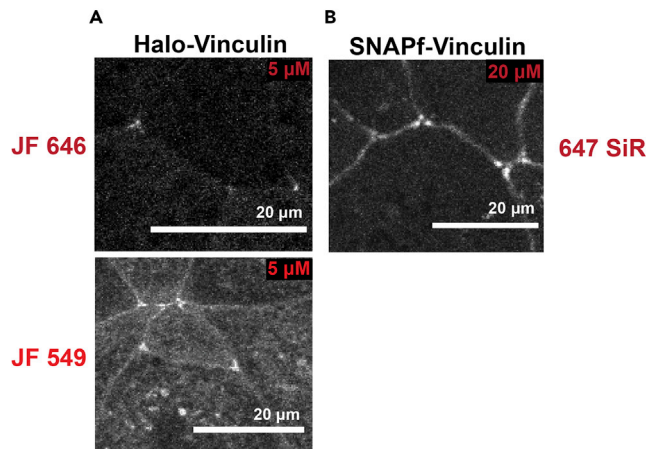


Figure 6. Microinjecting dye can label SNAPf/Halo-Vinculin *in vivo*

(A) Images of *Xenopus* embryos expressing Halo-Vinculin (10 pg) labeled via dye microinjection with Janelia Fluor 646 HaloTag (5 μ M) and Janelia Fluor 549 HaloTag (5 μ M).

(B) Image of *Xenopus* embryo expressing SNAPf-Vinculin (10 pg) labeled via dye microinjection with SNAP-Cell 647 SiR (20 μ M).

Scale Bars: 20 μ m.

- If the embryos are unhealthy, they may not be optimally expressing the SNAP- or Halo-tagged protein of interest, leading to low signal (step 22). It may be necessary to repeat the experiment with the same dye parameters, to determine whether the weak signal is an issue of embryo health vs. dye concentration or dye introduction (step 9 or step 16).

Note: If embryo health is a concern, we recommend using embryos collected from two different frogs as well as injecting a traditional fluorescent protein-tagged mRNA as a control for embryo health and efficient protein expression.

- Optimization of dye and/or mRNA concentration is needed.
 - It may take several experiments to optimize the best dye concentration for a new SNAP- or Halo-tagged protein of interest. It can be advantageous to test a variety of dye concentrations within the same experiment to help narrow down the optimal concentration for visualizing a protein (Figure 9) (step 9 or step 16). Altering the concentration of mRNA injected into the embryos can also be considered (step 11 or step 14). Avoiding an overexpression phenotype is a concern when exogenously expressing fusion proteins. The brightness of SNAP- and Halo-dyes helps to mitigate these concerns because dye concentration can be increased to improve visualization, rather than increasing the amount of mRNA. Finally, if the signal is not bright enough despite increasing the dye concentration, and you are co-injecting mRNA and dye (step 10), you might try injecting the dye and mRNA separately. The dyes are not RNase-free, so the RNA could potentially be degraded by the dye during injections (Campos et al., 2011).
- The dye has degraded.
 - After receiving SNAP- or Halo-dyes, we resuspended them in DMSO and aliquoted them into 0.5 mL tubes with lids wrapped in parafilm and stored them at -20°C . Storing the dyes in this manner gives the dyes a several month-long shelf life, with dye brightness decreasing over the length of storage (Figure 10). The recommended storage method entails desiccating the dyes after aliquoting and storing them at -20°C (Promega, 2013). This method should be utilized to extend the longevity of dyes, as well as maintain their brightness. Using the

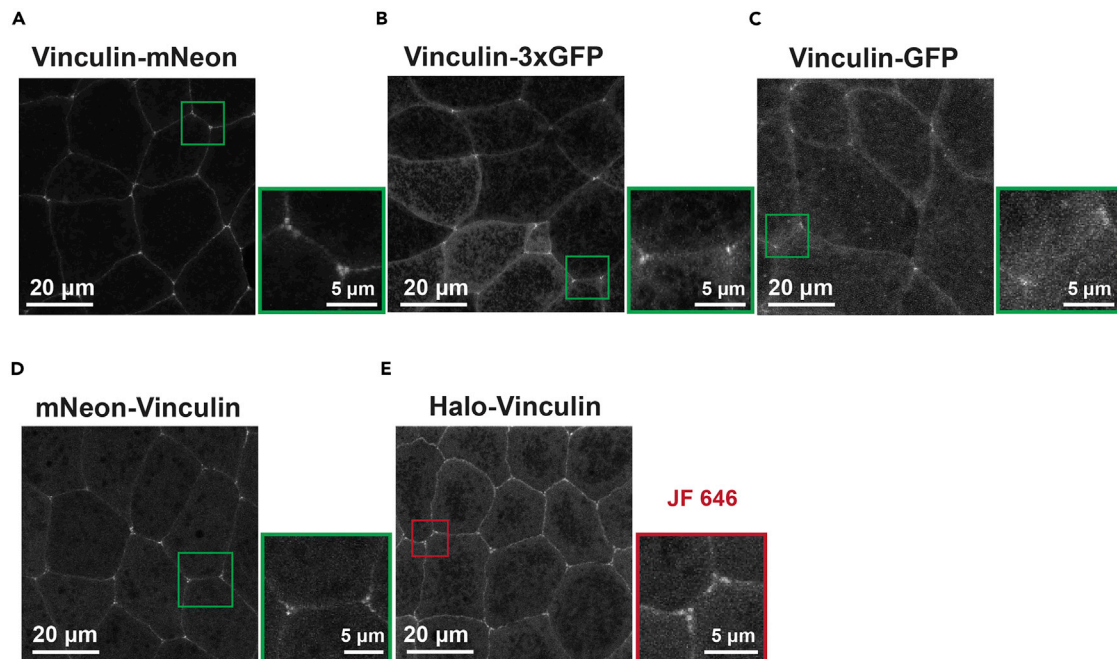


Figure 7. Halo-tag improves the visualization of difficult to image proteins compared with traditional fluorescent tags
(A–E) Images of *Xenopus* embryos expressing (A) Vinculin-mNeon (10 pg), (B) Vinculin-3xGFP (30 pg), (C) Vinculin-GFP (10 pg), (D) mNeon-Vinculin (10 pg), and (E) Halo-Vinculin (10 pg) labeled with microinjected *Janelia Fluor 646* HaloTag (5 μM). Scale Bars: 20 μm and 5 μm.

recommended storage method should help the dyes to maintain their integrity, reducing the need for excess dye purchases.

Problem 2

Signal is too bright.

Potential solution

- Optimization of dye and/or mRNA concentration is needed.
 - If the signal appears to be too bright, reducing the concentration of dye can help to decrease the signal (step 9 or step 16). The concentration of mRNA injected can also be reduced (Figure 9) (step 11 or step 14). Additionally, microscope laser power may need to be adjusted to very low settings to avoid signal saturation (step 22).

Problem 3

Dye Aggregation.

Potential solution

- Dye concentration is too high or embryos are unhealthy.
 - The embryos may form dye aggregates as a result of too high of a dye concentration or poor embryo quality. Decreasing the concentration of dye introduced may reduce the number of aggregates (Figure 9) (step 9 or step 16).
- The dye being used is not compatible with *Xenopus* embryos.

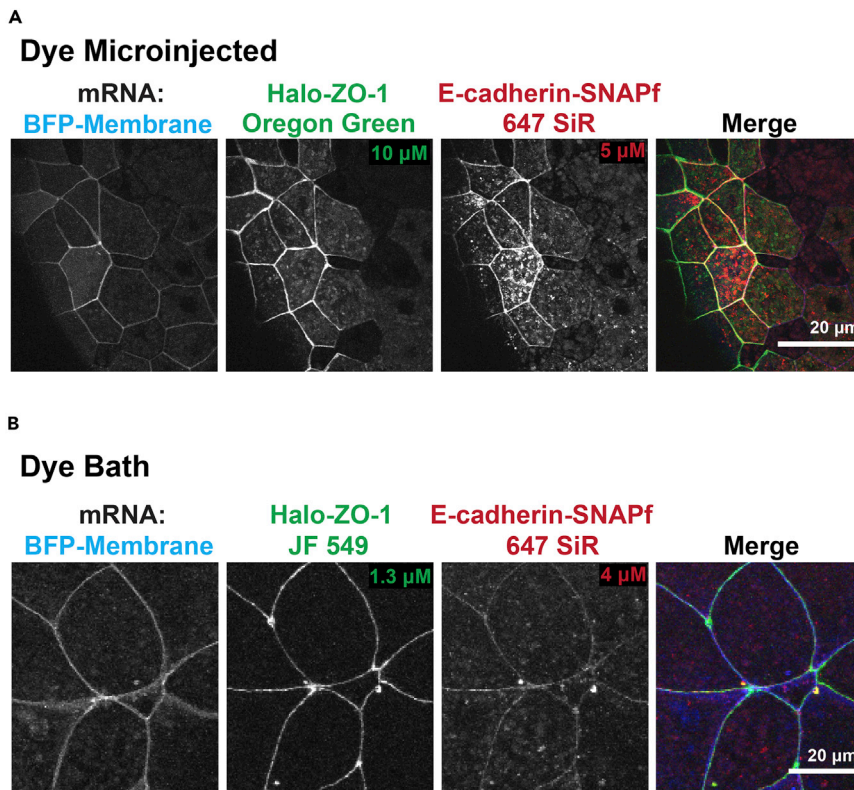


Figure 8. SNAP- and Halo-tagged proteins can be visualized simultaneously, through dye microinjections or dye baths, in gastrula-stage *Xenopus laevis* embryos

(A) *Xenopus laevis* gastrula-stage embryos expressing BFP-membrane (15 pg) (blue), Halo-ZO-1 (100 pg) labeled with microinjected Oregon Green HaloTag (10 μ M) (green), and E-cadherin-SNAPf (50 pg) labeled with microinjected SNAP-Cell 647 SiR (5 μ M) (red).

(B) *Xenopus laevis* gastrula-stage embryos expressing BFP-membrane (15 pg) (blue), Halo-ZO-1 (100 pg) labeled via dye bath with Janelia Fluor 549 HaloTag (1.3 μ M) (green), and E-cadherin-SNAPf (50 pg) labeled via dye bath with SNAP-Cell 647 SiR (4 μ M) (red).

Scale Bars: 20 μ m.

- Trying a different dye, specifically a “no-wash” dye, may reduce dye aggregates. In our hands, the Janelia Fluor “no-wash” dyes appear to exhibit a lower degree of aggregation than other commercially-available dyes.
- The dye being used may not be soluble in MMR at the concentration required for visualization.
 - During the dye optimization process, the concentrations tested were all below the published solubility limits of the dyes in water. However, the actual solubility limits of the dyes may be lower in MMR than in water, due to the presence of added salts. Therefore, lower dye concentrations could be used to mitigate solubility issues.

Problem 4

Dye microinjection or dye baths are incompatible with experimental workflow.

Potential solution

- Try a different dye introduction method not outlined in this protocol.
 - This protocol describes the use of dye microinjection or dye baths as methods for dye introduction to label SNAP- and Halo-tagged proteins of interest. These techniques may not be practical for all applications in *Xenopus* embryos. Concerns may also arise regarding the length of

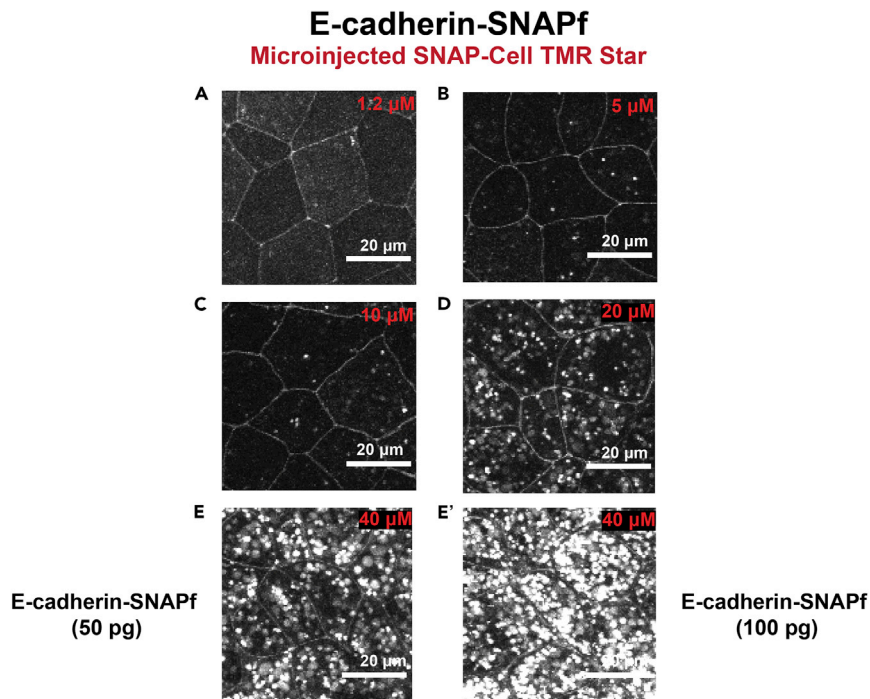


Figure 9. Dye and mRNA concentration optimization is necessary to reduce non-specific signal

(A–E) Images of E-cadherin-SNAPf (50 pg) labeled with SNAP-Cell TMR Star ((A) 1.2 μM , (B) 5 μM , (C) 10 μM , (D) 20 μM , (E) 40 μM) through dye microinjections.

(E') E-cadherin-SNAPf (100 pg) labeled with 40 μM SNAP-Cell TMR Star.

Scale Bars: 20 μm .

exposure to the dyes. There are potentially other avenues for dye introduction. Injecting dye into the blastocoel is one potential method for short-term dye exposure. For example, in [Stephenson et al. \(2019\)](#), blastocoel injections were used to introduce FluoZin-3, a small cell-impermeable fluorescent dye whose signal significantly increases upon binding zinc. Alternatively, by using *Xenopus* explants (flat, no vitelline envelope), dye could be added using a similar method to that of traditional SNAP- and Halo-dye introduction protocols for cell culture. Furthermore, explants could allow for pulse-chase style experiments ([Erdmann et al., 2019](#); [Van Itallie et al., 2017, 2019](#)).

Problem 5

The commercially available dyes are cost prohibitive.

Potential solution

- Use dye microinjections when possible.
 - One of the benefits of the SNAP- and Halo- tagging systems is that the tags are not labeled by a genetically expressed fluorescent protein, allowing for greater experimental flexibility. However, the SNAP- and Halo-tag dyes must then be purchased based on desired experimental parameters. One aliquot of purchased dye can be used for multiple experiments; however, dye costs can quickly add up when buying different colors. Dye microinjection allows for a smaller amount of dye to be used. If dye cost is a concern, we recommend using dye microinjections instead of dye baths to reduce cost. Preparing dye aliquots strategically, based on experimental parameters, can also mitigate dye waste (step 9). Finally, there are currently freely-available dyes through the Lavis Lab ([Lavis, 2021](#)).

E-cadherin-SNAPf SNAP-Cell TMR Star (40 μ M)

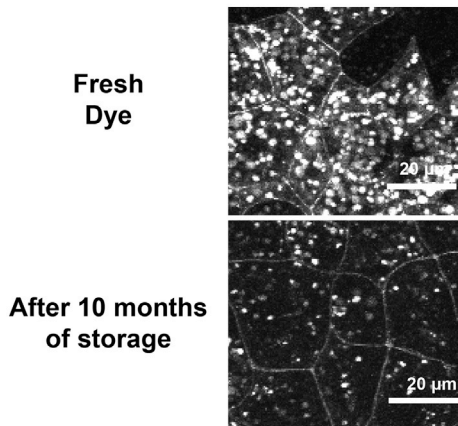


Figure 10. Non-desiccated SNAP-Cell TMR-Star dye aliquots can lose brightness following long-term storage

E-cadherin-SNAPf (50 μ g) labeled with SNAP-Cell TMR Star (40 M) (newly acquired, top) (long term storage, bottom). Scale Bars: 20 μ m.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ann L. Miller (annlm@umich.edu).

Materials availability

Empty SNAP- and Halo-tagged vectors generated for this study have been deposited with Addgene (pCS2+/N-SNAPf, 184415; pCS2+/C-SNAPf, 184416; pCS2+/N-Halo, 184417; pCS2+/C-Halo, 184418). Other DNA constructs utilized in this study are available upon request from the [lead contact](#).

Data and code availability

This study did not generate any datasets or code that have been deposited in a repository.

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AUTHOR CONTRIBUTIONS

C.E.D., L.v.d.G., and A.L.M. conceptualized the study; C.E.D. and L.v.d.G. developed the methodology with input from A.L.M.; L.v.d.G. made the pCS2+/N- & C-SNAPf constructs; C.E.D. made the pCS2+/N- & C- Halo constructs; C.E.D. performed the majority of experiments and wrote the original draft of the manuscript; L.v.d.G. conducted the experiments for [Figure 7](#); all authors revised the manuscript; A.L.M. acquired funding and supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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