Title: Data associated with “Improved methods for single-molecule fluorescence in situ hybridization and immunofluorescence in Caenorhabditis elegans embryos”

Abstract: Visualization of gene products in Caenorhabditis elegans has provided insights into the molecular and biological functions of many novel genes in their native contexts. Single-molecule Fluorescence In Situ Hybridization (smFISH) and Immunofluorescence (IF) visualize the abundance and localization of mRNAs and proteins, respectively, allowing researchers to elucidate the localization, dynamics, and functions of the corresponding genes. Whereas both smFISH and immunofluorescence have been foundational techniques in molecular biology, each protocol has challenges in the C. elegans embryo. smFISH protocols suffer from high initial costs and can photobleach rapidly. Immunofluorescence requires technically challenging permeabilization and slide preparation. Most importantly, published smFISH and IF protocols have predominantly been mutually exclusive, preventing the exploration of relationships between an mRNA and a relevant protein in the same sample. Here, we describe protocols to perform immunofluorescence and smFISH in C. elegans embryos either in sequence or simultaneously. We also present protocols to perform smFISH or immunofluorescence alone, including several improvements and optimizations to existing approaches. These protocols include 1) improved fixation and permeabilization steps to preserve cellular morphology while maintaining probe and antibody accessibility in the embryo, 2) a streamlined, in-tube approach for antibody staining that negates freeze-cracking, 3) a previously validated protocol to perform the cost-reducing smiFISH (single molecule inexpensive FISH) adaptation, 4) slide preparation using empirically determined optimal antifade products, and 5) straightforward quantification and data analysis methods. Figure 1 shows a schematic workflow for each protocol. Finally, we discuss tricks and tips to help the reader optimize and troubleshoot individual steps in each protocol. Together, these protocols and optimizations simplify existing protocols for single-molecule RNA and protein detection. Moreover, simultaneous, high-resolution imaging of proteins and RNAs of interest will permit analysis, quantification, and comparison of protein and RNA distributions, furthering our understanding of the relationship between RNAs and their protein products or cellular markers in early development.

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Recommended data citation: Parker, D. 2021. Dataset associated with “Improved methods for single-molecule fluorescence in situ hybridization and immunofluorescence in Caenorhabditis elegans embryos”. Colorado State University. Libraries. http://dx.doi.org/10.25675/10217/233940

Associated article citation: Parker, D. M., Winkenbach, L. P., Parker, A., Boyson, S., & Nishimura, E. O. (2021). Improved methods for single-molecule fluorescence *in situ* hybridization and immunofluorescence in *Caenorhabditis elegans* embryos. *Current Protocols*, 1, e299. https://doi.org/10.1002/cpz1.299

Format of data files: .tar.bz2, .tiff, .zip, .csv, .ijm (ImageJ macro), .py, .txt

Description: This repository contains the data set associated with the *Current Protocols* publication, "Improved methods for single-molecule fluorescence in situ hybridization and immunofluorescence in Caenorhabditis elegans embryos." This data includes the raw images associated with each figure, the complete data sets for both antifade and buffer quantification, and sample output data from both quantification pipelines. This README file describes each file in more detail, including which embryos from the raw data were analyzed and how files were generated.

The scale for every image in this repository is 9.3301 pixels/micron. Each image was obtained using a GE DeltaVision Elite microscope with a 60x objective. The only post-processing performed on each image shown in the repository is the conversion from the proprietary .D3V file format to .tiff format and compression using bzip2 and tar. Further details on fluorescent microscopy can be found in the methods of https://doi.org/10.1242/dev.186817. Images shown in figures **were not deconvolved**.

File list:

nos-2_set-3_n-propyl_and_vecta_antifade.tar.bz2 (2.663Gb): Contains antifade data associated with figure 5 for n-propyl glycol and VECTASHIELD antifades probing for *nos-2* and *set-3* RNAs.

imb-2_erm-1_all_antifade.tar.bz2 (4.940Gb): Contains antifade data associated with figure 5 for the combination of all antifades probing for *imb-2* and *erm-1* RNAs.

nos-2_set-3_no_antifade.tar.bz2 (2.373Gb): Contains antifade data associated with figure 5 for FISH performed with no antifades probing for *nos-2* and *set-3* RNAs.

imb-2_erm-1_diamond_and_n-propyl_antifade.tar.bz2 (4.935Gb): Contains antifade data associated with figure 5 for ProLong Diamond and n-propyl gallate antifades probing for *imb-2* and *erm-1* RNAs.

imb-2_erm-1_n-propyl_and_vecta_antifade.tar.bz2 (4.763Gb): Contains antifade data associated with figure 5 for n-propyl gallate and VECTASHIELD antifades probing for *imb-2* and *erm-1*.

nos-2_set-3_vecta_antifade_only.tar.bz2 (3.274Gb): Contains antifade data associated with figure 5 for VECTASHIELD antifade alone probing for *nos-2* and *set-3* RNAs.

IF-FISH_repository_without_antifade.tar.bz2 (8.970Gb): Contains all data associated with the manuscript, excluding the antifade experiments (Data associated with Figure 5)

nos-2_set-3_n-propyl_antifade_only.tar.bz2 (3.140Gb): Contains antifade data associated with figure 5 for n-propyl gallate antifade alone probing for *nos-2* and *set-3* RNAs.

imb-2_erm-1_diamond_antifade_only.tar.bz2 (4.793Gb): Contains antifade data associated with figure 5 for ProLong Diamond antifade alone probing for *imb-2* and *set-3* RNAs.

imb-2_erm-1_n-propyl_antifade_ony.tar.bz2 (4.954Gb): Contains antifade data associated with figure 5 for n-propyl gallate antifade alone probing for *imb-2* and *erm-1* RNAs.

nos-2_set-3_diamond_antifade_only.tar.bz2 (2.690Gb): Contains antifade data associated with figure 5 for ProLong Diamond antifade alone probing for *nos-2* and *set-3* RNAs.

nos-2_sets-3_all_antifades.tar.bz2 (2.779Gb): Contains antifade data associated with figure 5 for the combination of all antifades probing for *nos-2* and *set-3* RNAs.

nos-2_set-3_diamond_and_n-propyl_antifade.tar.bz2 (2.865Gb): Contains antifade data associated with figure 5 for ProLong Diamond and n-propyl gallate probing for *nos-2* and *set-3* RNAs.

nos-2_set-3_diamond_and_vecta_antifade.tar.bz2 (2.687Gb): Contains antifade data associated with figure 5 for ProLong Diamond and VECTASHIELD antifades probing for *nos-2* and *set-3* RNAs.

imb-2_erm-1_diamond_and_vecta_antifade.tar.bz2 (4.679Gb): Contains antifade data associated with figure 5 for ProLong Diamond and VECTASHIELD antifades probing for *imb-2* and *erm-1* RNAs.

imb-2_erm-1_vecta_antifade_only.tar.bz2 (5.272Gb): Contains antifade data associated with figure 5 for VECTASHIELD antifade alone, probing for *imb-2* and *erm-1* RNAs.

imb-2_erm-1_no_antifade.tar.bz2 (2.088Gb); Contains antifade data associated with figure 5 for FISH with no antifade probing for *imb-2* and *erm-1* RNAs.

Figure 2: Immunofluorescence

N2_k76-1-20_antiMouseAF488-1-250.tiff: Shows an embryo stained for endogenous PGL-1 using Support Protocol 1. Mouse K76 (PGL-1) antibody was used for primary staining at a 1:20 dilution. Anti-mouse Alexa Fluor 488 secondary antibody (Jackson ImmunoResearch, cat. No. 115-545-003) was used to fluorescently label PGL-1 bound K76 primary antibody in channel 1. DAPI was used to visualize DNA in channel 2.

N2_p-granule-no-primary_antiMouseAF488-1-250.tiff: Shows an embryo stained only using anti-mouse Alexa Fluor 488 secondary antibody (Jackson ImmunoResearch, cat. No. 115-545-003) in channel 1. DAPI was used to visualize DNA in channel 2.

N2_2A4-1-1000_antiMouseAF488-1-250.tiff: Shows an embryo stained for endogenous ELT-2 using Support Protocol 1. Mouse 2A4 (ELT-2) antibody was used for primary staining at a 1:1000 dilution. Anti-mouse Alexa Fluor 488 secondary antibody (Jackson ImmunoResearch, cat. No. 115-545-003) was used to fluorescently label ELT-2 bound 2A4 primary antibody in channel 1. DAPI was used to visualize DNA in channel 2.

N2_elt-no-primary_antiMouseAF488-1-250.tiff: Shows an embryo stained only using anti-mouse Alexa Fluor 488 secondary antibody (Jackson ImmunoResearch, cat. No. 115-545-003) in channel 1. DAPI was used to visualize DNA in channel 2.

Figure 3: smFISH vs smiFISH

imb-2-smFISH-670_smiFISH-FLAPY-610.tiff: Shows an N2 embryo probing for *imb-2* mRNA using both smFISH and smiFISH. smFISH probes were used to label *imb-2* with Quasar 670 in channel 1. smiFISH probes utilizing the FLAP Y sequence were used to label *imb-2* with Cal Fluor 610 in channel 2. DAPI is shown staining nuclei in channel 3.

nos-2-smiFISH-FLAPY-670_smFISH-610.tiff: Shows an N2 embryo probing for *nos-2* mRNA using both smFISH and smiFISH. smFISH probes were used to label *nos-2* with Cal Fluor 610 in channel 2. smiFISH probes utilizing the FLAP Y sequence were used to label *nos-2* with Quasar 670 in channel 1. DAPI is shown staining nuclei in channel 3.

Figure 4: Buffer Comparison

The data presented in this figure was generated by analyzing a series of smFISH micrographs generated in N2 worms. Under the parent folder, there are two subfolders: imb-2_erm-1 and nos-2_set-3. These folders represent the transcripts visualized in each experiment with *imb-2* and *nos-2* being visualized in channel 1 using Quasar 670 labeled smFISH probes and *erm-1* and *set-3* being visualized in channel 2 using Cal Fluor

610 labeled smFISH probes. DAPI is labeled in all images in channel 3. In each of these subdirectories is another pair of folders titled “Homebrew” or “Stellaris,” indicating which buffer condition was utilized. In these folders are the images that were quantified to create figure 4. The image title corresponds to the identical entry in supplementary table 4. Notes follow only for images where multiple embryos were visualized to describe which embryo was quantified. If there are multiple embryos in an image, but clipped by the frame, only the embryo fully in frame was analyzed.

Figure_4_buffer_comparison/imb-2_erm-1/Stellaris/210127_Image_05.tiff: The embryo closest to the bottom left was quantified.

Figure_4_buffer_comparison/imb-2_erm-1/Stellaris/210209_Image_01.tiff: The embryo on the left was quantified.

Figure_4_buffer_comparison/imb-2_erm-1/Stellaris/210209_Image_05.tiff: The embryo on the right was quantified.

Figure_4_buffer_comparison/imb-2_erm-1/Stellaris/210214_Image_04.tiff: The 2-cell embryo on the middle-left was quantified.

Figure_4_buffer_comparison/nos-2_set-3/Homebrew/210209_Image_03.tiff: Both 4-cell embryos fully in frame were quantified.

Figure_4_buffer_comparison/nos-2_set-3/Homebrew/210221_Image_07.tiff: The embryo on the upper right was quantified.

Figure_4_buffer_comparison/nos-2_set-3/Stellaris/210209_Image_01.tiff: The embryo in the center was quantified.

Figure_4_buffer_comparison/nos-2_set-3/Stellaris/210209_Image_05.tiff: The embryo on the bottom left was quantified.

Figure_4_buffer_comparison/nos-2_set-3/Stellaris/210221_Image_02.tiff: The embryo to the left was quantified.

Figure 5: Antifade Comparison

The data presented in this figure was generated by analyzing a series of smFISH micrographs generated in PH::GFP (LP306) worms. Under the parent folder, there are two subfolders: imb-2_erm-1 and nos-2_set-3. These folders represent the transcripts visualized in each experiment with *imb-2* and *nos-2* being visualized in channel 1 using Quasar 670 labeled smFISH probes and *erm-1* and *set-3* being visualized in channel 2 using Cal Fluor 610 labeled smFISH probes. PH::GFP is visualized in channel 3. DAPI is labeled in all images in channel 4. Transilluminated light is shown in channel 5. Under each of these subdirectories are several folders named after the antifade each embryo preparation was incubated in after performing the smFISH protocol (Support Protocol 2.) The image title corresponds to the identical entry in supplementary tables 5 and 6. Notes follow only for images where multiple embryos were visualized to describe which embryo was quantified. If there are multiple embryos in an image, but clipped by the frame, only the embryo fully in frame was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/All/200103_Image_05.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/All/200110_Image_01.tiff: The embryo in the center was analyzed

Figure_5_antifade_comparison/imb-2_erm-1/All/200110_Image_03.tiff: The embryo on the middle left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/All/200110_Image_04.tiff: The embryo on the bottom was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_only/200103_Image_05.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_only/200110_Image_03.tiff: The embryo on the bottom left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_only/200110_Image_04.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_only/200110_Image_05.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_Vecta/200103_Image_01.tiff: The embryo on the upper left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_Vecta/200103_Image_05.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_Vecta/200110_Image_04.tiff: The embryo on the top was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Diamond_Vecta/200311_Image_05.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_diamond/200103_Image_01.tiff: The embryo on the top was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_diamond/200103_Image_03.tiff: The embryo on the bottom left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_only/200103_Image_06.tiff: The embryo on the left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_only/200103_Image_08.tiff: The 4-cell embryo towards the upper right was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_only/200103_Image_09.tiff: The 4-cell embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_only/200110_Image_01.tiff: The 4-cell embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_only/200311_Image_01.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200103_Image_01.tiff: The 4-cell embryo in the center was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200103_Image_02.tiff: The embryo on the left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200103_Image_03.tiff: The embryo on the top was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200103_Image_04.tiff: The 6-cell towards the right was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200103_Image_05.tiff: The bottom embryo was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200110_Image_01.tiff: The 4-cell embryo on the left was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200110_Image_02.tiff: The 4-cell embryo on top was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/n-propyl_vecta/200110_Image_03.tiff: The 4-cell embryo on the right was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/No_antifade/210524_Image_01.tiff: Both embryos were analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/No_antifade/210524_Image_02.tiff: Both embryos were analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/No_antifade/210526_Image_01.tiff: Both embryos were analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/No_antifade/210526_Image_02.tiff: Both embryos were analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Vecta_only/200110_Image_05.tiff: The top embryo was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Vecta_only/200110_Image_06.tiff: The center embryo was analyzed.

Figure_5_antifade_comparison/imb-2_erm-1/Vecta_only/200311_Image_09.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/All/200306_Image_01.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/All/200306_Image_02.tiff: The center embryo was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/All/200306_Image_03.tiff: The upper embryo was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/All/200306_Image_04.tiff: The upper embryo was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/Diamond_only/200217_Image_04.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/Diamond_only/200217_Image_05.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/Diamond_only/200306_Image_02.tiff: The upper embryo was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/Diamond_only/200306_Image_03.tiff: The embryo on the left was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/Diamond_Vecta/200306_image_02.tiff: The embryo on the right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/Diamond_Vecta/200306_image_03.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_diamond/200217_Image_03.tiff: Both embryos were analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_diamond/200217_Image_04.tiff: The embryo in the bottom-center was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_diamond/200306_Image_01.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_diamond/200306_Image_02.tiff: The embryo on the bottom left was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_diamond/200306_Image_03.tiff: The embryo in the upper right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_diamond/200306_Image_05.tiff: The embryo on the bottom right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_only/200217_Image_02.tiff: The embryo on the bottom left was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_only/200306_Image_01.tiff: The embryo in the center was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_only/200306_Image_02.tiff: The 4-cell on the upper left was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_only/200306_Image_03.tiff: The 4-cell towards the upper middle was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_vecta/200217_Image_03.tiff: The embryo on the bottom left was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_vecta/200217_Image_06.tiff: The embryo on the bottom was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_vecta/200306_Image_01.tiff: The embryo on the upper right was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/n-propyl_vecta/200306_Image_05.tiff: The bottom embryo was analyzed.

Figure_5_antifade_comparison/nos-2_set-3/No_antifade/210521_Image_03.tiff: Both embryos were analyzed.

Figure_5_antifade_comparison/nos-2_set-3/No_antifade/210526_Image_01.tiff: The embryo in the center was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200217_Image_03.tiff: The embryo on the right was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200306_Image_01.tiff: The upper embryo was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200306_Image_02.tiff: The embryo towards the upper right was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200306_Image_03.tiff: The embryo towards the upper center was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200306_Image_04.tiff: The embryo on the left was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200306_Image_05.tiff: The embryo on the middle right was quantified.

Figure_5_antifade_comparison/nos-2_set-3/Vecta_only/200306_Image_06.tiff: The embryo in the center was quantified.

Figure 6: Sequential Immunofluorescence and smFISH

N2_2A4-ELT-2-1-1000_anti_mouse-1-250_elt-2-RNA.tiff: This image shows the data used to generate Figure 6C. The presented embryo is the uppermost one in the image. In channel 1, *elt-2* RNA was imaged using Cal Fluor 610 labeled smFISH probes. In channel 2, ELT-2 protein was stained using 1:1000 anti-ELT-2 2A4 primary antibody and 1:250 anti-mouse AlexaFluor 488 secondary antibody. In channel 3 DAPI illuminates DNA.

N2_K76-PGL-1-1-20_anti-mouse-1-250_nos-2-RNA_cpg-2-RNA.tiff: This image shows the data used to generate Figure 6A and 6B. The presented embryo is the one on the right in the image. In channel 1, *nos-2* RNA was imaged using Quasar 670 labeled smFISH probes. In channel 2, *cgp-2* RNA was labeled using Cal Fluor 610 labeled smFISH probes. In channel 3, PGL-1 protein was stained using 1:20 anti-PGL-1 k76 primary antibody and 1:250 anti-mouse AlexaFluor 488 secondary antibody. In channel 3 DAPI illuminates DNA.

Figure 7: Simultaneous Immunofluorescence and smFISH

PATR-1-GFP-no-nanobody-nos-2_RNA_cpg-2-RNA.tiff: This image shows the data used to generate the bottom panel of figure 7. In channel 1, *nos-2* RNA was imaged using Quasar 670 labeled smFISH probes. In channel 2, *cpg-2* RNA was imaged using Cal Fluor 610 labeled smFISH probes. In channel 3, PATR-1::GFP is imaged, demonstrating that the GFP signal is largely eliminated by smFISH fixation. In channel 4, DNA is labeled with DAPI.

PATR-1+nanobody_nos-2-RNA.tiff: This image shows the data used to generate the top panel of figure 7. In channel 1, *nos-2* RNA was imaged using Quasar 670 labeled smFISH probes. In channel 2, PATR-1::GFP is stained using Janelia Fluor 549 conjugated anti-GFP nanobodies. In channel 3, DNA is labeled with DAPI.

Antifade_analysis_sample: This folder contains the sample input, output, file structure, etc. used to generate the antifade comparison data. The subfolder No_antifade_nos-2_set-3_210524_Image_01 describes that this sample is taken from the no antifade data set imaging the nos-2 and set-3 transcripts. Within this folder, 210524_Image_01.tif is the raw image file. The C1 and C2 subfolders, as well as the .tif files starting with C3 and C4 were generated by the Split_image_for_antifade_analysis.ijm macro, which splits the image file by channels. This macro then stores the FISH data (*nos-2* in C1 and *set-3* in C2) in their own subfolders, but leaves the other channels (PH::GFP in C3 and DAPI in C4) in this directory. Within the C1 and C2 subfolders, the .tif file represents the smFISH data for the respective transcript. The .zip file contains the outline used as a region of interest by the antifade_quantification.ijm macro to generate the results.csv file. The .zip file can be opened as a region of interest in FIJI by dragging it into the ROI manager. The results.csv contains information on the exposure #, the ROI area, the mean intensity of the ROI, and the minimum and maximum intensity of the ROI. These .csv files were used to generate the quantification plotted in figure 5.

Signal_to_noise_analysis_sample: This folder contains the sample input, output, file structure, etc. used to generate the buffer comparison data. The subfolder Stellaris_nos-2_set-3_210209_Image_05 describes the this sample data was taken from an experiment using Stellaris buffers to image *nos-2* and *set-3*. As with the antifade analysis, the subfolders were generated by splitting the raw .tif image, N2_Stellaris_nos-2_set-3_210209_Image_05.tif, and putting FISH data (C1 = *nos-2*, C2 = *set-3*) in their own subfolder and leaving the DAPI data in the parent folder. In each subfolder the .tif file is the individual FISH channel analyzed. These files were run through FISH-quant which generates the __outline.txt file to demonstrate the region of interest under which RNA spots were detected, the __settings_MATURE.txt file to save the spot detection setting used to detect RNAs, and the __spots.txt file to record the coordinates of each RNA spot. The __spots.txt files were then converted to __spots.csv files using the ZYX_spot_converter.py script to make them compatible with the ImJoy SNR calculator plugin. These .csv files were then analyzed to create the __SNR_all.csv files, which were used to generate the figure.