This document is the README file for the supplemental material to the manuscript *Improved methods for protein and single-molecule RNA detection in Caenorhabditis elegans embryos*. This document will describe the methods used to quantify data as well as the contents of each supplemental file.

**Quantification of signal-to-noise ratio:** Signal-to-noise ratio (SNR) was calculated in figure 4 to determine whether Stellaris or homemade buffers result in better image quality. This was achieved by first analyzing the RNA content of each embryo using the Matlab implementation of FISHquant. Once RNA spots had been detected, the custom Python script ZYX\_spot\_converter.py was used to extract RNA spot coordinate data from the FISHquant output \_\_spots.txt files and convert it into a form compatible with the ImJoy Signal-to-Noise (SNR Calculation) plugin. This plugin can be found at <https://imjoy.io/#/app?w=fq-main&plugin=fish-quant%2Ffq-imjoy%3ASNR-calculation%40stable>. The plugin was then run on each image and corresponding spot coordinate data file to determine the signal-to-noise ratio of each RNA detected in the embryo. A sample output file is included in this supplemental packet as SNR\_quantification\_sample.csv. Once the SNR had been calculated for each image, the custom python script SNR\_tabulator.py was used extract the file name, buffer condition, transcript imaged, average snr, standard deviation of snr for each spot, and the 95% confidence interval of the average SNR. The output of this script is included as Table\_S4\_Signal\_to\_noise\_quantification\_table.csv and was used to generate figure 4 using the custom .R script Buffer\_quant.Rmd.

**Quantification of antifade optimality:** Several combinations of antifade were tested in Figure 5 to determine the optimal antifade mixture for use with Cal Fluor 610 and Quasar 670 labeled smFISH probes in *C. elegans* embryos. The quantification of mean fluorescence intensity for each embryo was performed using the custom FIJI macros Split\_image.ijm and antifade\_quantification.ijm. The Split\_image.ijm macro selected a rectangular region of interest from smFISH data and split the channels before saving them in a file structure compatible with downstream analysis. Each smFISH channel was then quantified using the antifade\_quantification.ijm macro, which measures the average fluorescence intensity under a region of interest through a time course of images. The region of interest and quantifaction results are then saved to the same folder as the image as a .zip file and a .csv file respectively. A sample folder illustrating the file hierarchy and outputs of these macros are provided in Antifade\_sample\_data. The average intensity data for each embryo, transcript, and antifade condition were then agglomerated using the custom python script Antifade\_quantification\_table\_maker.py. The output from this script is presented as Antifade\_all\_data.csv. The data were then summarized using the Antifade\_summary\_table\_maker.py script, which produced the Antifade\_data\_summarized.csv file. Statistical analysis was then performed using the Antifade\_statistics.py script, which produced the Antifade\_statistics.csv file. Finally, the Antifade\_quantification.R script was used to generate the plots in figure 5.

**Table S1. Worm Strains**

This table denotes the strains of *C. elegans* used in this study.

**Table S2. Antibodies**

This table denotes the antibodies used in this study.

**Table S3. smFISH and smiFISH probes**

This table denotes the smFISH and smiFISH oligos used in this study.

**Table S4. Signal to noise quantification table**

This file contains the tabulated data used to create figure 4, a comparison of Stellaris and homebrew buffers.

**Table S5. Antifade all data**

This file contains the tabulated data generated by comparing fluorescence decrease upon prolonged imaging using various antifades.

**Table S6. Antifade data reorganized**

This file contains the tabulated data from Table S5 reorganized with normalizations added.

**Table S7. Antifade data statistics**

This file contains the antifade data from Table S6 summarized with statistical analysis included. This table was used to generate Figure 5.