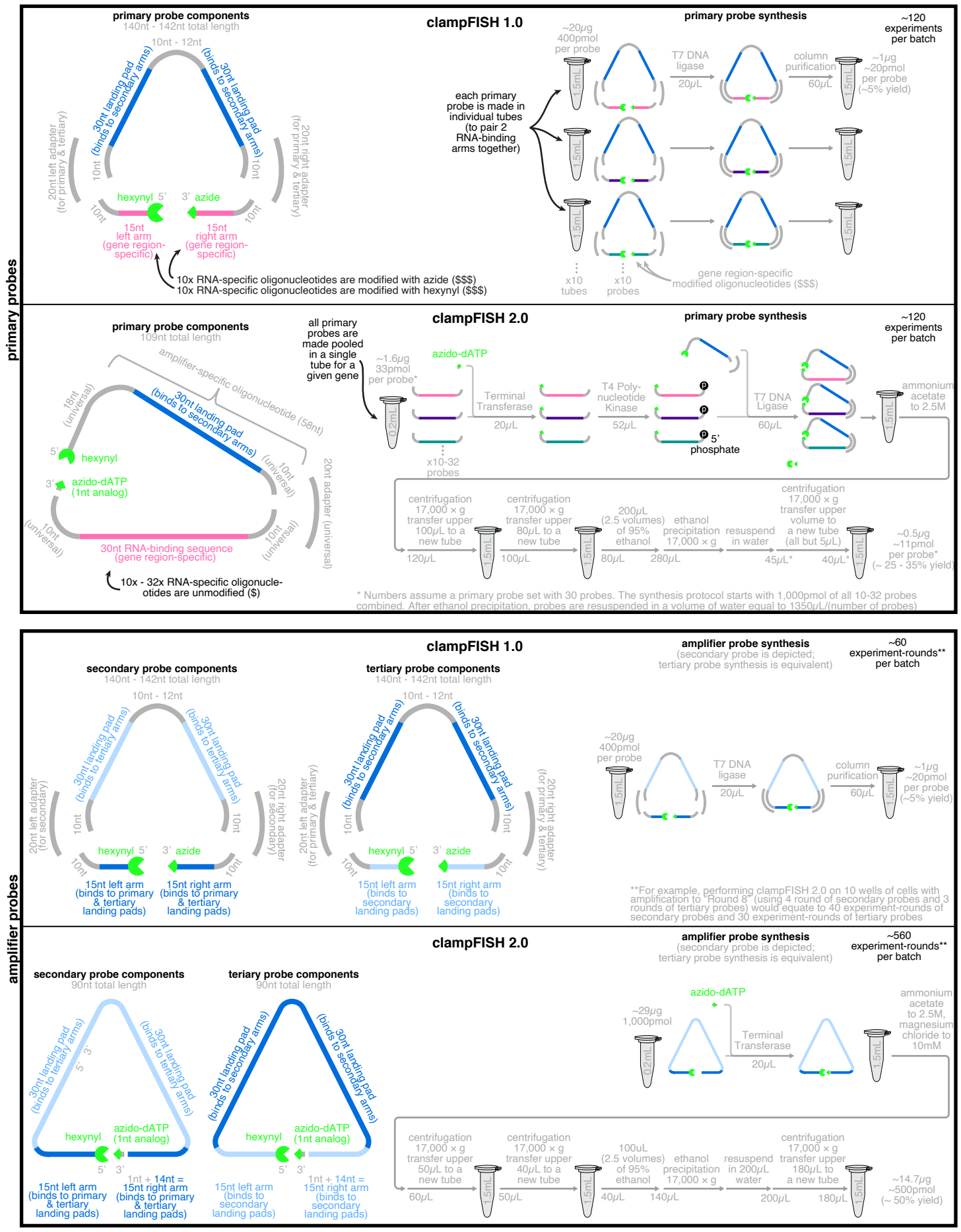
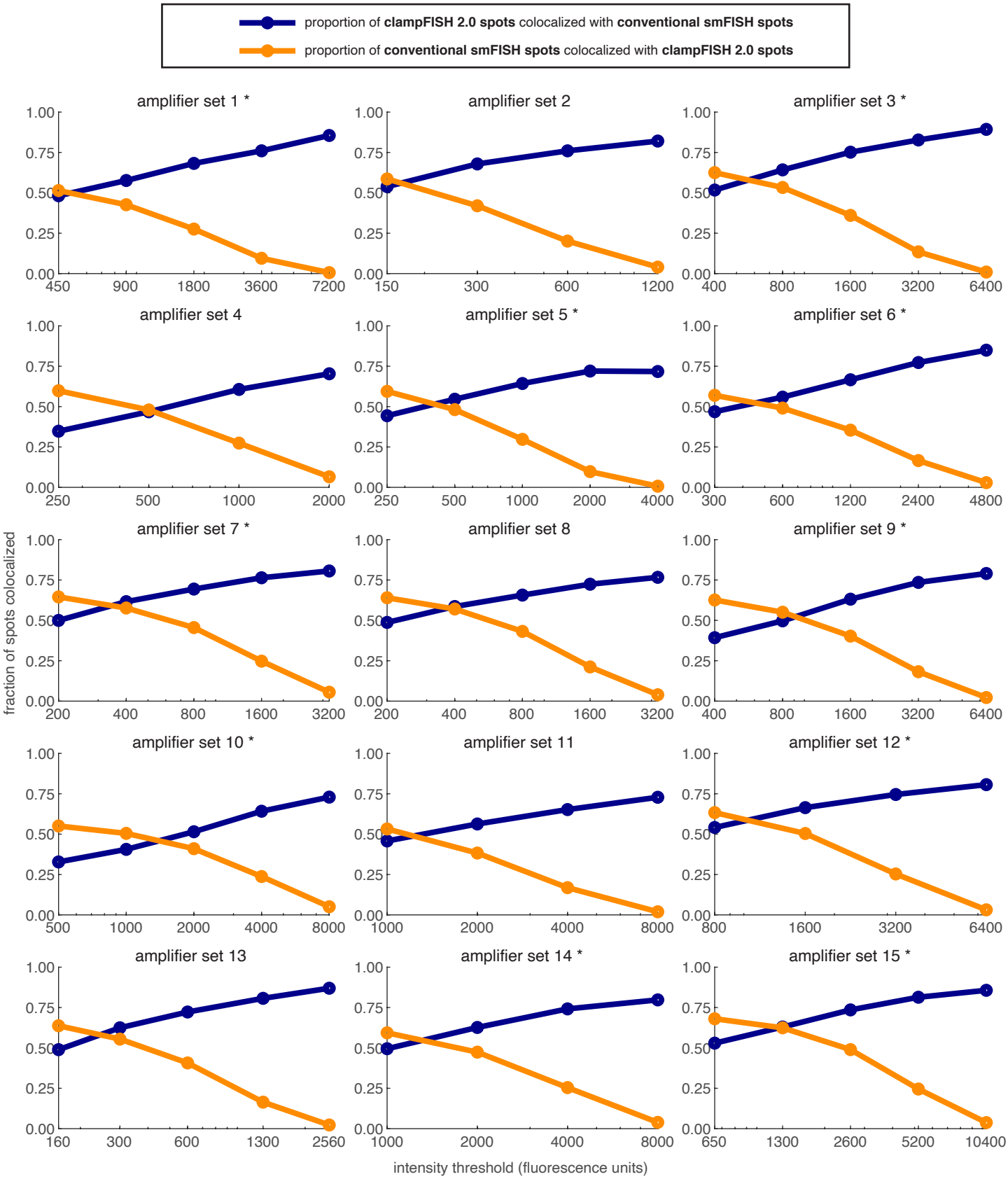
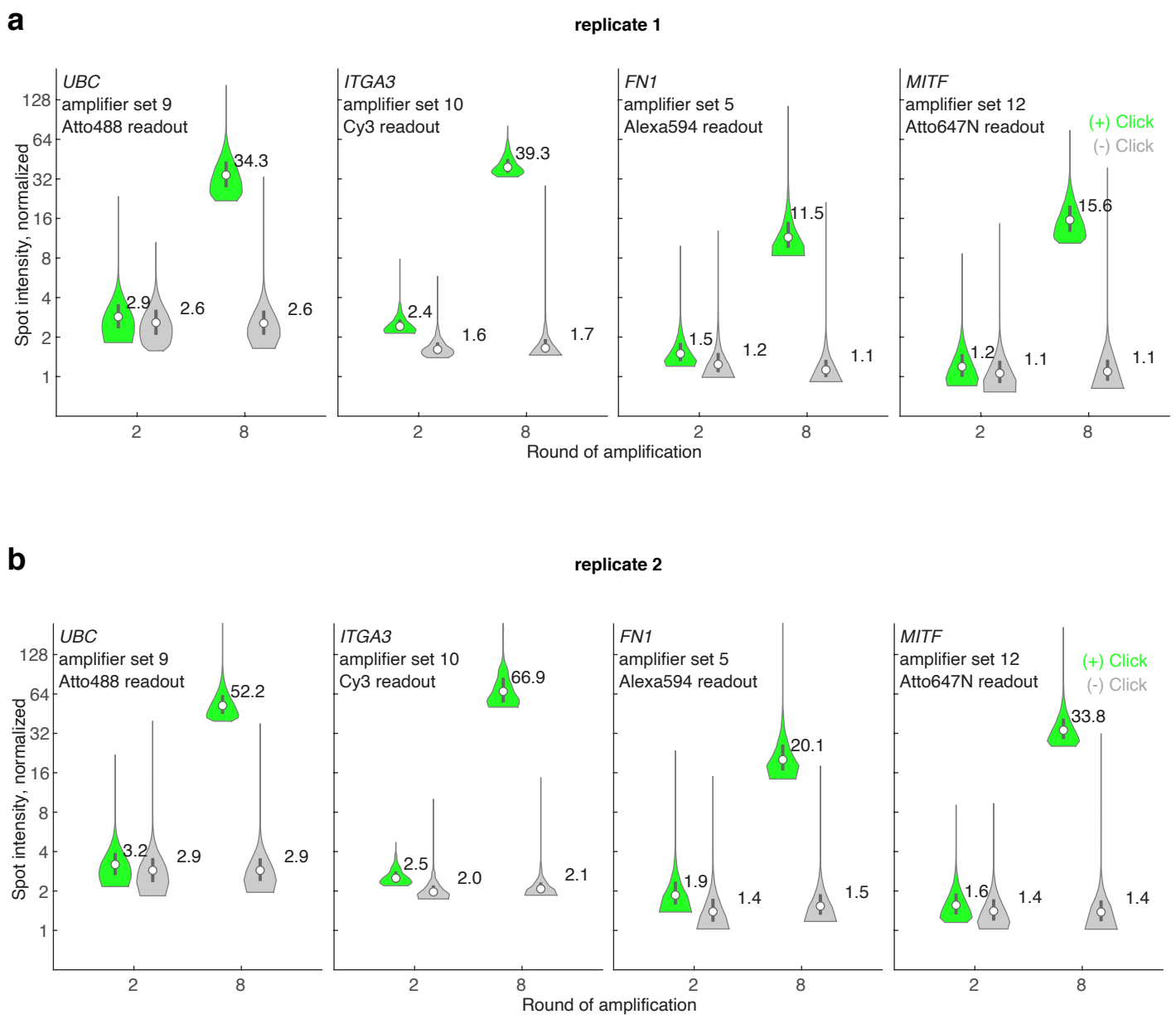


Supplementary Figure 1: Schematic of clampFISH 1.0 (top) and clampFISH 2.0 (bottom) probe synthesis protocols.

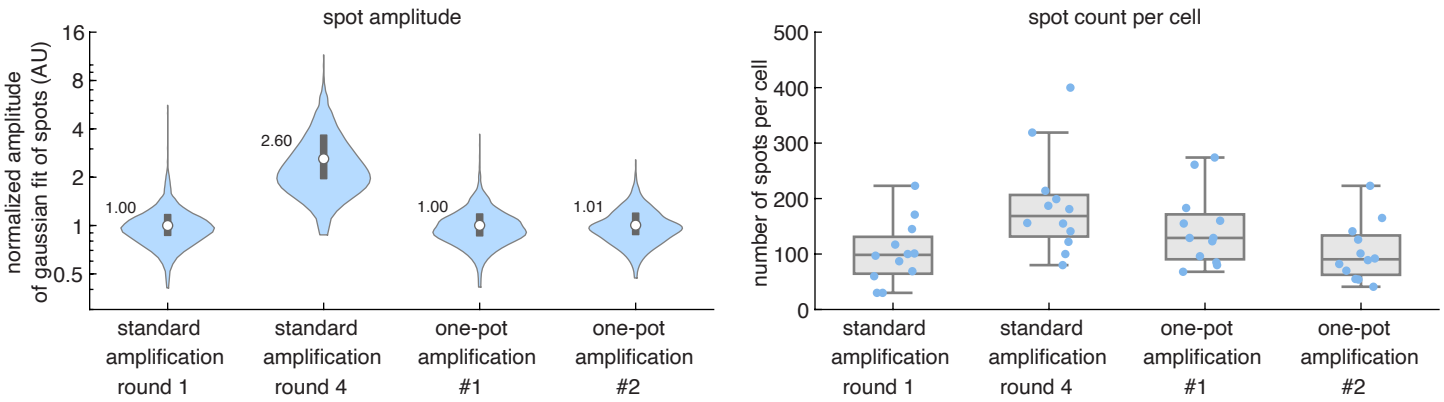


Supplementary Figure 2: clampFISH 2.0 spots colocalize with conventional single-molecule RNA FISH (smFISH) spots when probing the same RNA. ClampFISH 2.0 (10 primary probes; readouts in Atto 647N) and conventional smFISH probes (15 probes; labeled in Alexa 555) were both designed to target non-overlapping regions of GFP mRNA (see Methods section description of 'amplifier screen' for details). ClampFISH 2.0 primaries were used with one of 15 amplifier sets (plots 1-15). To call conventional single-molecule RNA FISH spots we chose cell-specific manual intensity thresholds, whereas to call clampFISH 2.0 spots we chose a single threshold which was varied (x-axis). Shown are the fraction of clampFISH 2.0 spots co-localizing with conventional smFISH (blue) and the fraction of conventional smFISH spots co-localizing with clampFISH 2.0 spots (orange). Asterisks (*) mark the 10 amplifier sets that were used in later multiplexing experiments.



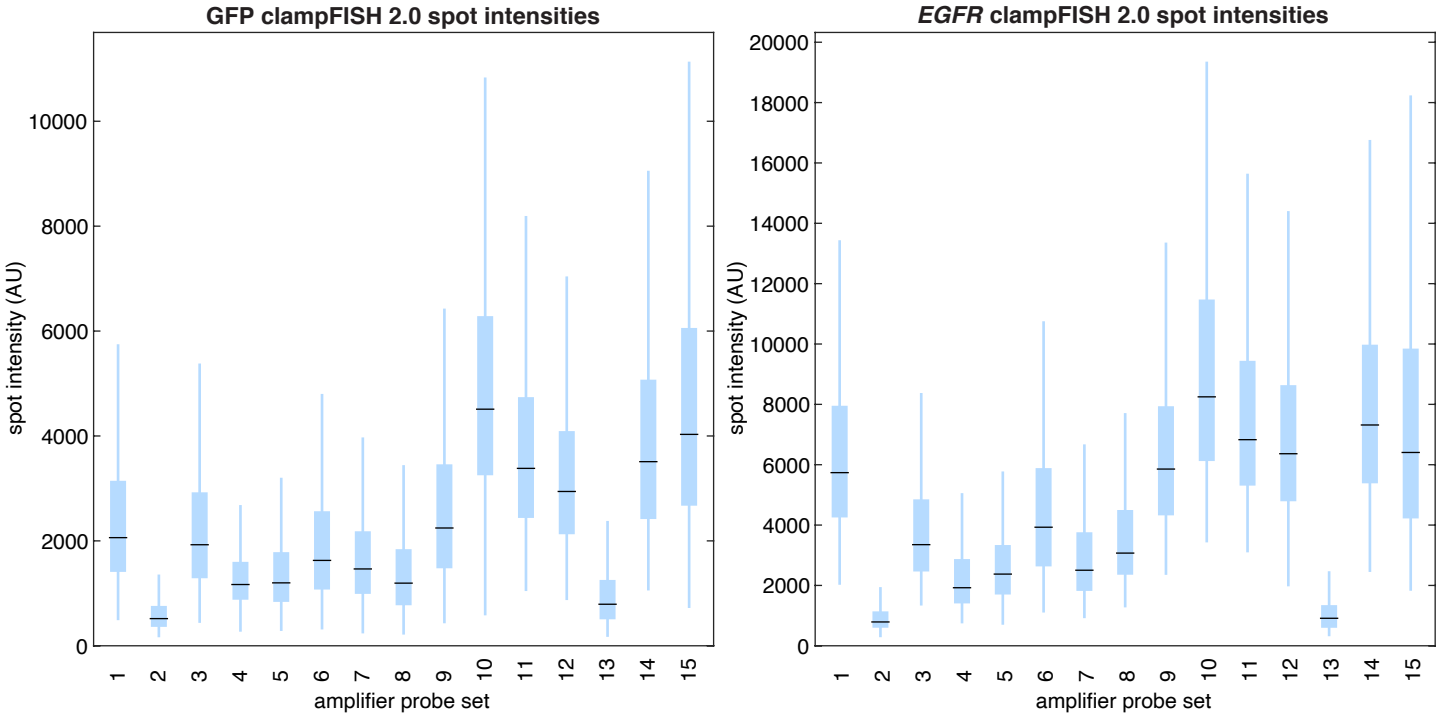
Supplementary Figure 3: clampFISH 2.0 signal amplification is dependent on the click reaction. (a) In an amplification characterization experiment, we performed the clampFISH 2.0 amplification steps to rounds 2 and 8, both with (green) and without (grey) the copper sulfate catalyst included in the click reaction (labeled values are median intensities). **(b)** a biological replicate (different passage) of the same experiment as in (a).

We saw no amplification from round 2 to round 8 in the absence of the copper catalyst, confirming that the click reaction is an essential step for clampFISH 2.0. For spot counts associated with each condition in panels a-b, see Supplementary Table 12. Circles are median values and bounds of boxes are 25th and 75th percentiles.

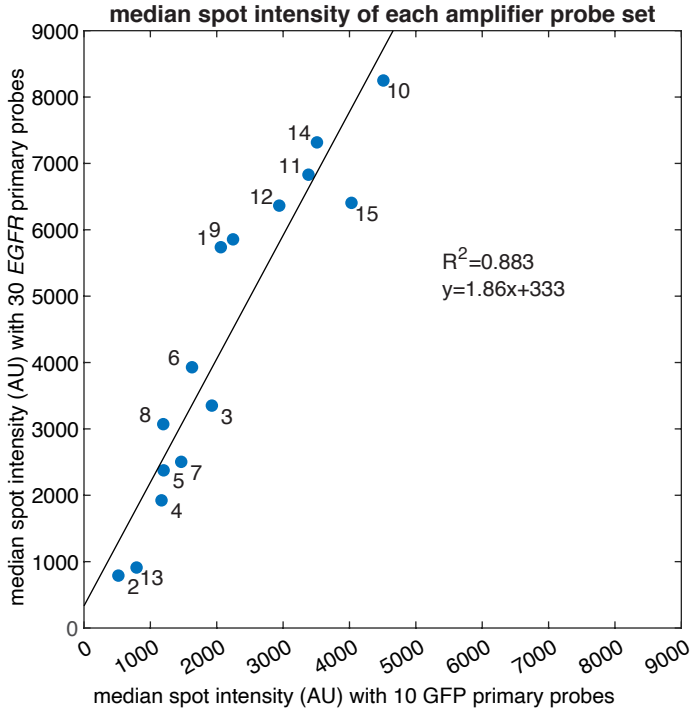


Supplementary Figure 4: A one-pot amplification protocol did not produce amplified spots. We tested whether a one-pot amplification protocol, where the secondary probes, tertiary probes, and click reagents were added simultaneously, could produce amplified spots. In one-pot amplification #1, we included 10% formamide and 10% dextran sulfate in the one-pot mixture, whereas one-pot amplification #2 did not have these reagents (see Methods section for details). We also performed clampFISH 2.0 in the standard manner to rounds 1 (Ie. only primary probes) and round 4. **Left**: Normalized amplitudes of the *MITF* clampFISH 2.0 spots, where circles and numbers shown are median values and bounds of boxes are 25th and 75th percentiles. n= 1230, 2254, 1743, and 1239 spots (from left to right). **Right**: *MITF* clampFISH 2.0 spot count per cell from 12 segmented cells per condition. Circles are median values, bounds of boxes are 25th and 75th percentiles, and whiskers extend to non-outlier minima and maxima, where data falling more than 1.5 times the interquartile range beyond the box bounds are considered outliers. The experiment was performed once.

The spot intensities of both one-pot conditions were not higher than the intensities produced by the primaries alone (round 1), indicating that the tested one-pot amplification conditions are not a viable alternative to the standard step-wise amplification protocol. The two other primary probes and amplifier sets tested (*FN1* with amplifier set 5, *NGFR* with amplifier set 1) also did not amplify in the one-pot conditions (data not shown).

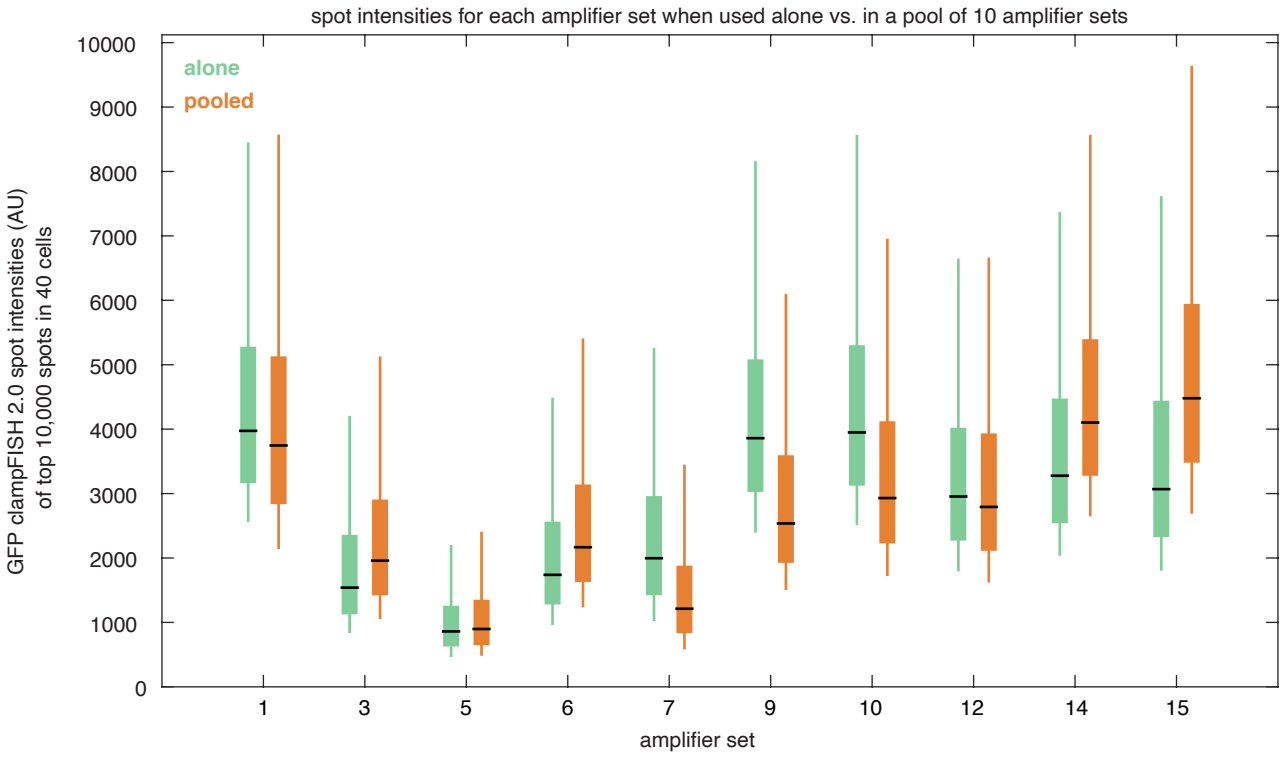


Supplementary Figure 5: A screen of amplifier probe sets revealed designs with a high level of signal amplification. In a screen for amplifier probe sequences, we tested 15 amplifier sets, each used with primary probes targeting GFP (left) and *EGFR* (right) amplified to round 8 (in total, 30 primary probe sets were used: 2 targets x 15 amplifier sets). We labeled the clampFISH 2.0 scaffolds with 20 nucleotide secondary-targeting readout probes (coupled to Atto 647N) and performed conventional single-molecule RNA FISH (GFP probes in Alexa 555, *EGFR* probes in Cy3) targeting non-overlapping regions of the same mRNA as the primary probes. We counted the number of conventional single-molecule RNA FISH spots in each segmented cell, took an equivalent number of the highest-intensity clampFISH 2.0 spots from that cell, and plotted these clampFISH 2.0 spot intensities. Center lines are median values, bounds of boxes are 25th and 75th percentiles, and whiskers extend to non-outlier minima and maxima, where data falling more than 1.5 times the interquartile range beyond the box bounds are considered outliers. 11,252 GFP and 881 *EGFR* outliers, out of 294,220 and 22,861 total points respectively, are not shown.

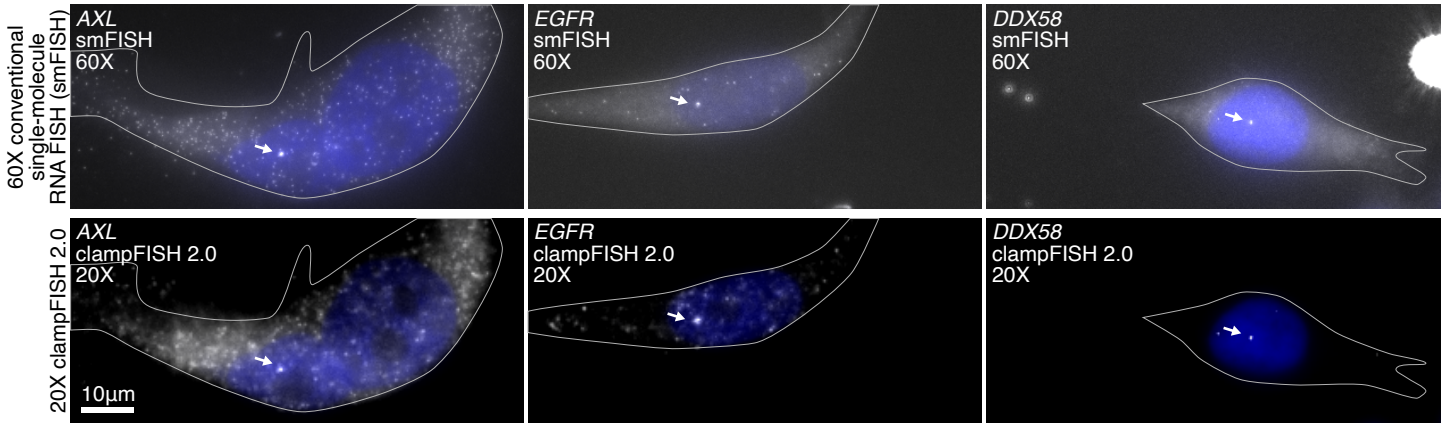


Supplementary Figure 6: Amplifier probe sets can modularly be used with various primary probe sets. We plotted the median spot intensity generated by each clampFISH 2.0 amplifier set (labeled 1 to 15) from the amplifier screen experiment when used with primaries targeting GFP (x-axis) or *EGFR* (y-axis).

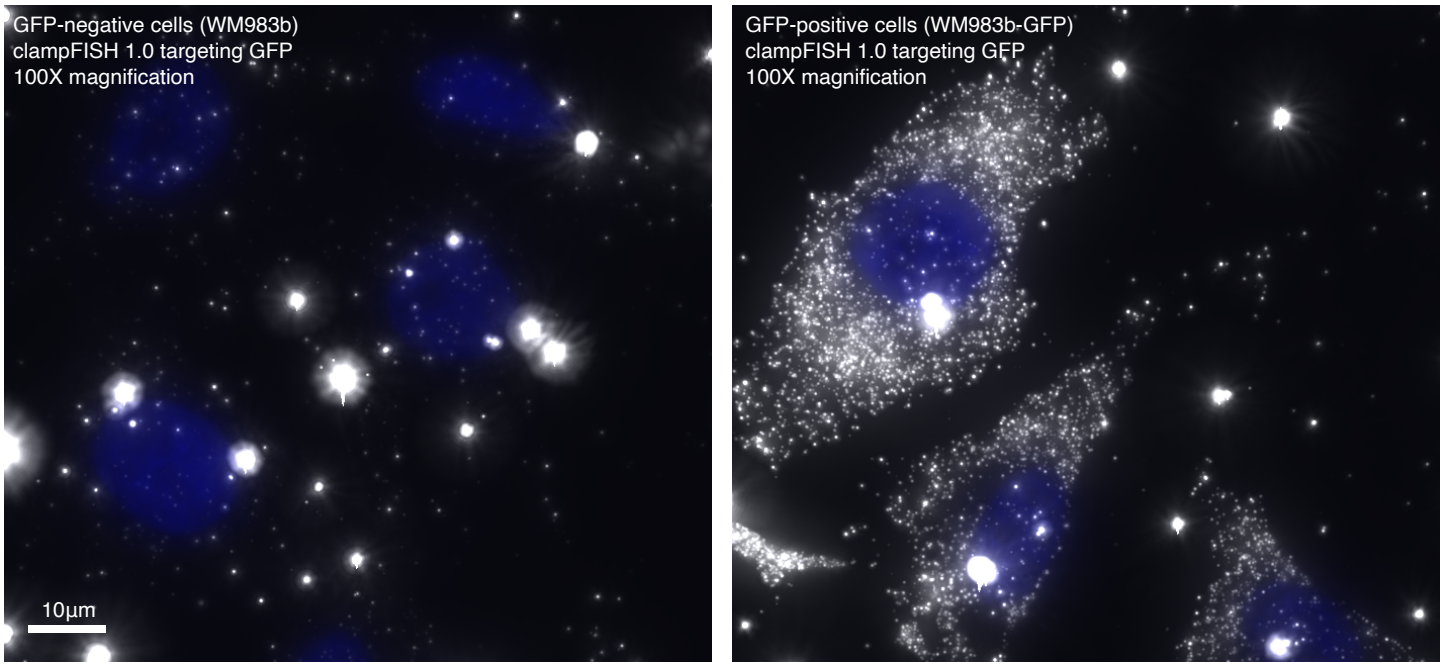
We observed a strong correlation (R^2=0.883) between the two primary probe sets, suggesting that gene-specific effects on amplification play a minimal role in their performance. The slope of the regression suggests a nearly 2-fold increase in spot intensities when amplifier sets were used with the *EGFR* probe set over the GFP probe set, likely as a result of the 3-fold higher number of primary probes (30 for *EGFR* vs. 10 for GFP).



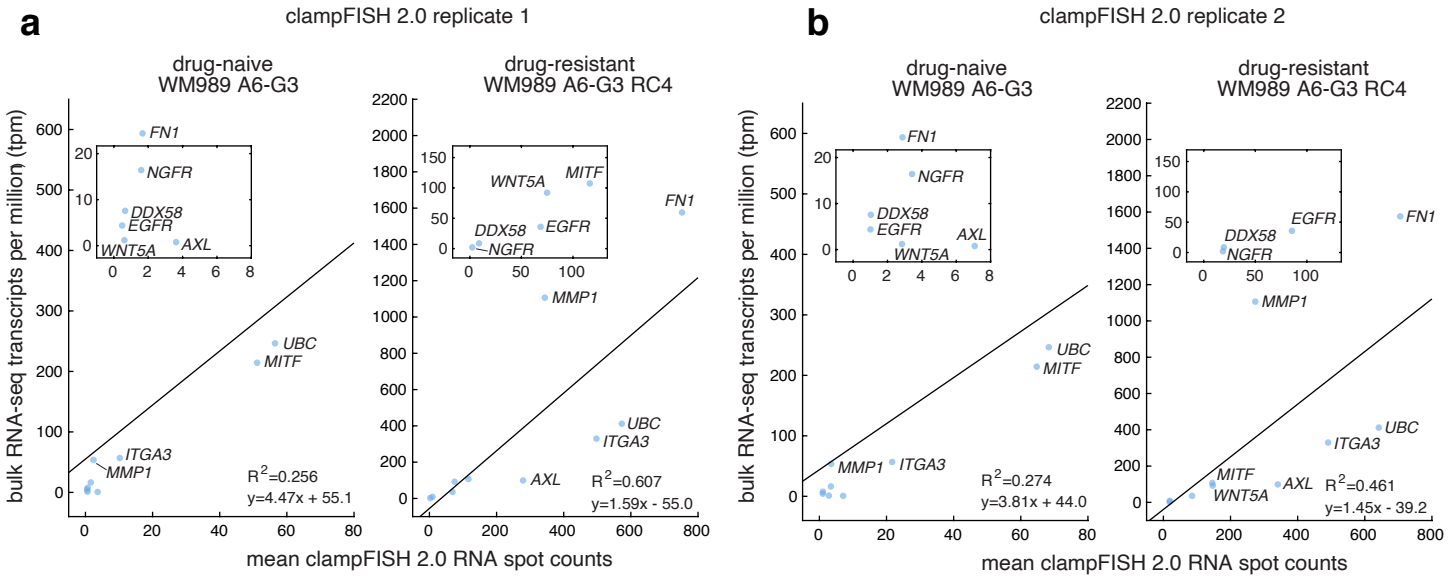
Supplementary Figure 7: Amplifier probe sets amplify signal similarly when used alone vs. when used in a pool of 10 amplifier probes. We hybridized 10 GFP-targeting primary probe sets, with each set ligated to a different amplifier-binding oligonucleotide, and amplified each in one of two ways: with its corresponding amplifier probe set alone (green) or with a pool of all 10 amplifier sets (orange). Plotted are the intensities of the 10,000 highest-intensity spots from 40 segmented cells per condition. Center lines are median values, bounds of boxes are 25th and 75th percentiles, and whiskers extend to non-outlier minima and maxima, where data falling more than 1.5 times the interquartile range beyond the box bounds are considered outliers. 379 'alone' spots outliers and 418 'pooled' spot outliers not shown.



Supplementary Figure 8: clampFISH 2.0 detects transcription sites. Conventional single-molecule RNA FISH (smFISH) (top row) and clampFISH 2.0 (bottom row) probing non-overlapping regions of the same RNAs (from left to right: AXL, EGFR, and DDX58) (see Methods section description of conventional single-molecule RNA FISH comparison experiment for details). Images of smFISH, labeled in Cy3, are maximum intensity projections of 5 z-planes at 0.5µm z-steps taken with a 60X objective with 2 second exposure times for all RNAs. Images of clampFISH 2.0 are from a single plane taken with a 20X objective with exposure times of 1 second (*AXL*, *DDX58*) or 500 milliseconds (*EGFR*), with readout probes labeled in Atto 647N (*AXL*, *EGFR*) and Alexa Fluor 594 (*DDX58*). Arrows point to putative transcription sites with multiple RNA copies, identified as such from their nuclear localization and their increased spot intensity relative to other nuclear and cytoplasmic RNA spots. The experiment was performed twice with similar results.

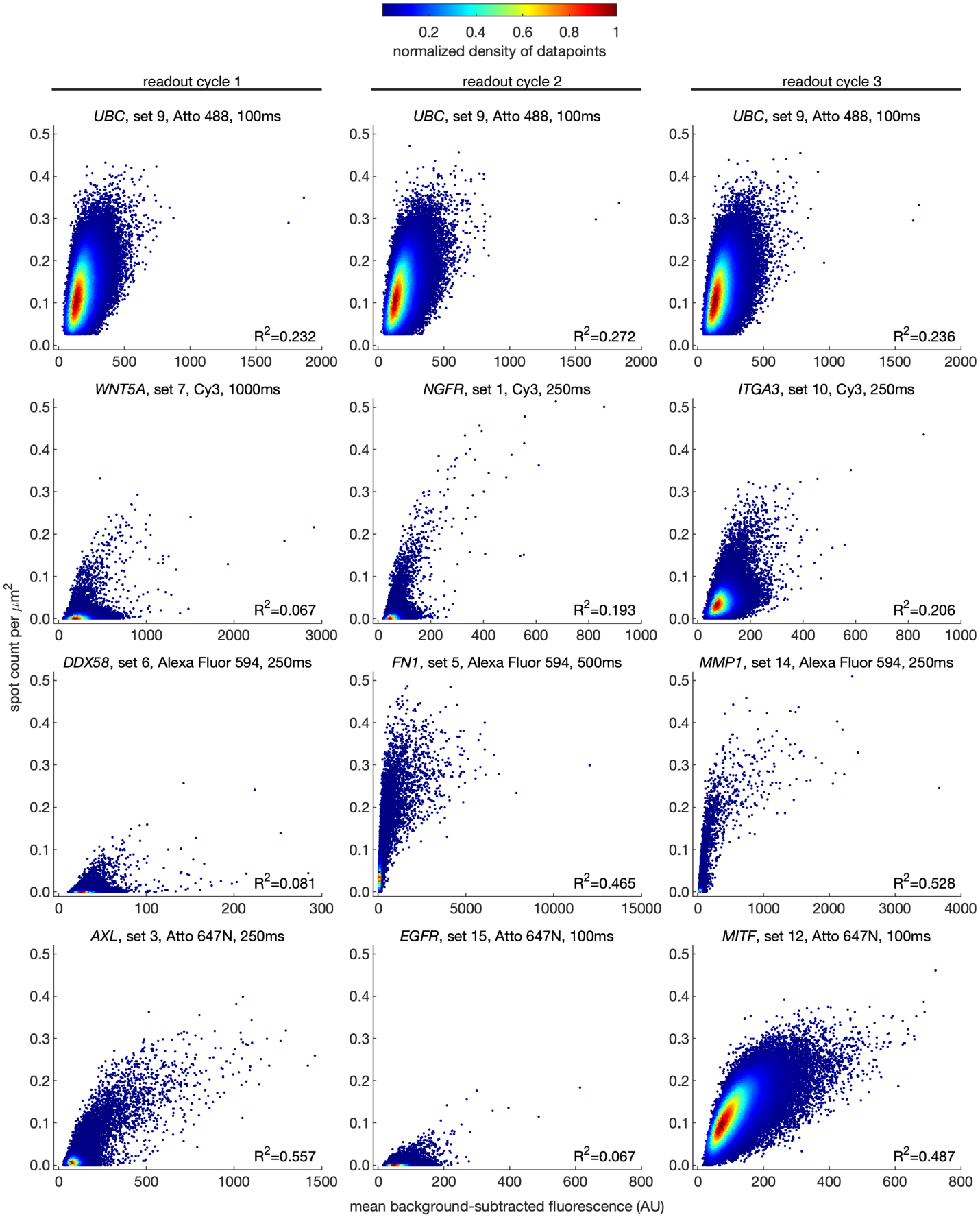


Supplementary Figure 9: Off-target spots seen in clampFISH 1.0 precluded the identification of transcription sites. Maximum intensity projections of 20 z-planes from 100X magnification image stacks of clampFISH 1.0 targeting GFP mRNA in cells without GFP (WM983b cells, left) and in cells expressing GFP (WM983b-GFP cells, right). Images are from Rouhanifard et al. 2018 (see that paper's Methods section titled "Comparison of amplification methods" for details). Data are typical for clampFISH 1.0 results from Rouhanifard et al. 2018, although the number of off-target spots can vary from experiment to experiment.

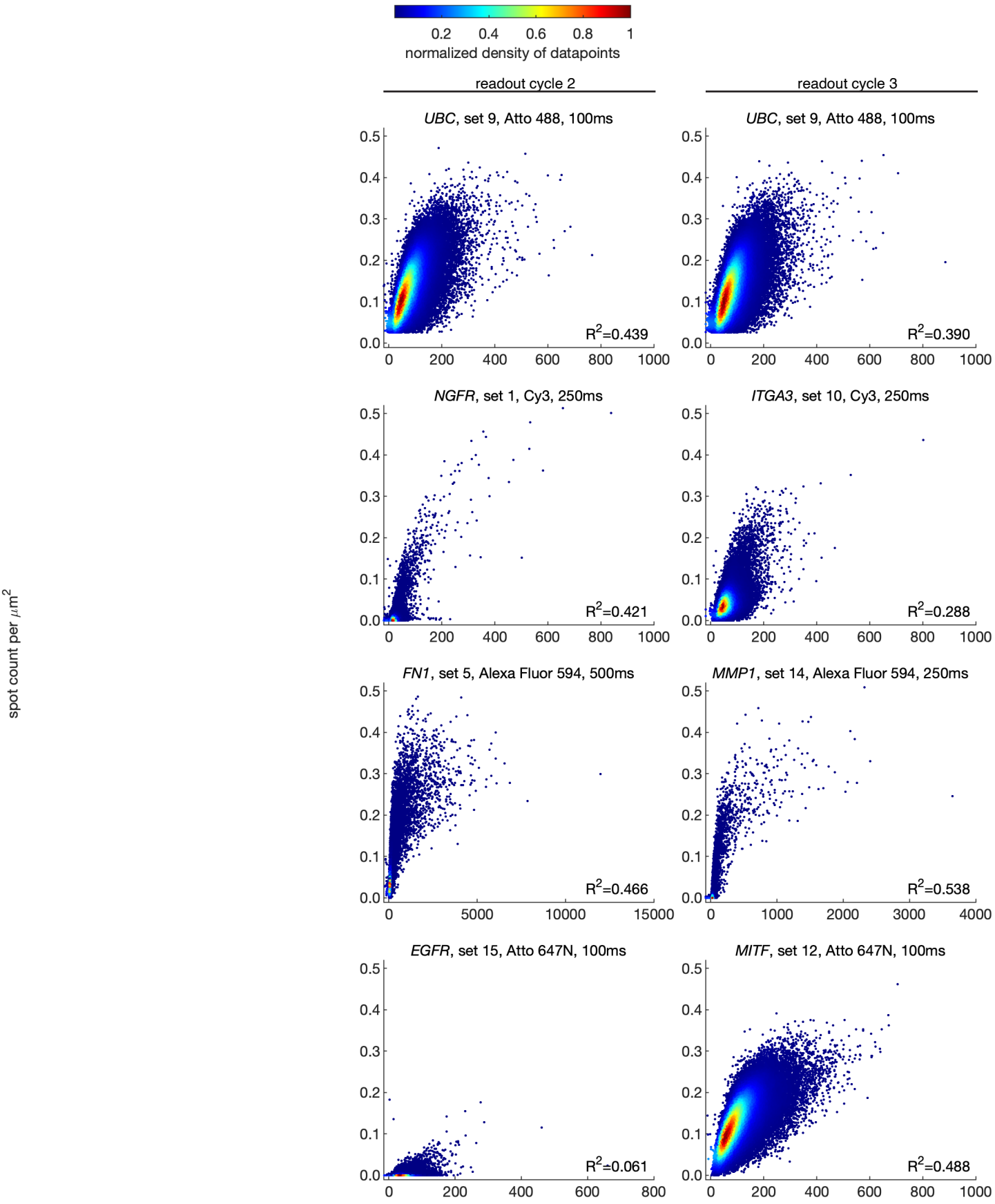


Supplementary Figure 10: Mean clampFISH 2.0 spot counts are correlated with bulk RNA sequencing data. (a) Mean clampFISH 2.0 spot counts from 722,298 drug-naive WM989 A6-G3 cells (left) and 2,155 vemurafenib-resistant WM989 A6-G3 RC4 cells (right) for the 10 genes from the high-throughput profiling experiment (x-axis) and bulk RNA-seq transcripts per million (y-axis) for each of the two cell lines. **(b)** A technical replicate of the same experiment as in (a), but with data from 234,410 drug-naive WM989 A6-G3 cells and 5,150 vemurafenib-resistant WM989 A6-G3 RC4 cells, using the same bulk RNA sequencing data.

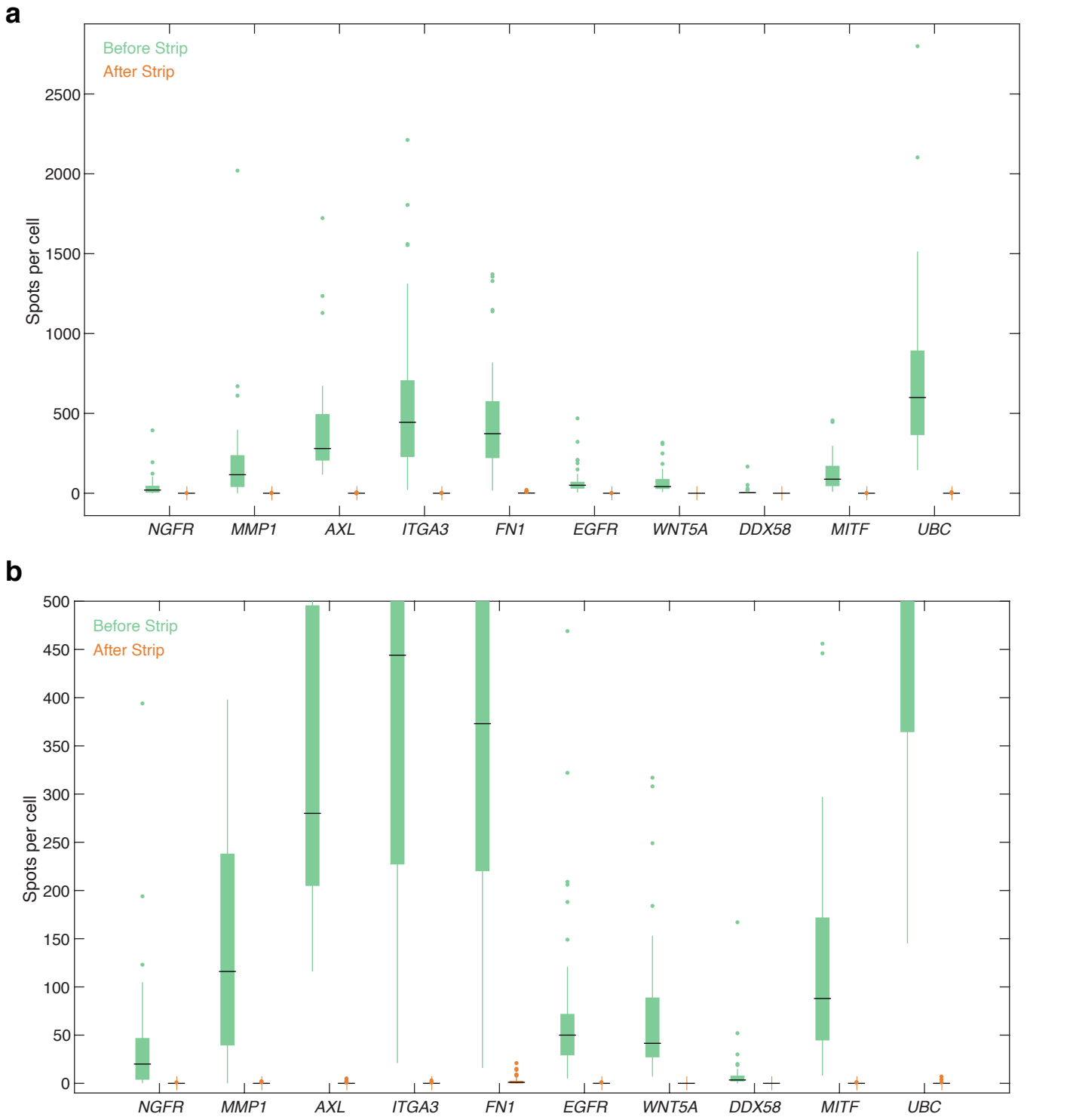
We observed that *FN1* and *MMP1*, both of which have a lower mean clampFISH 2.0 spot count than would be expected from the remaining genes' trend, are expressed at particularly high levels in a subset of cells (see Fig. 3b), suggesting that optical crowding at 20X magnification may contribute to their under-counting by clampFISH 2.0.



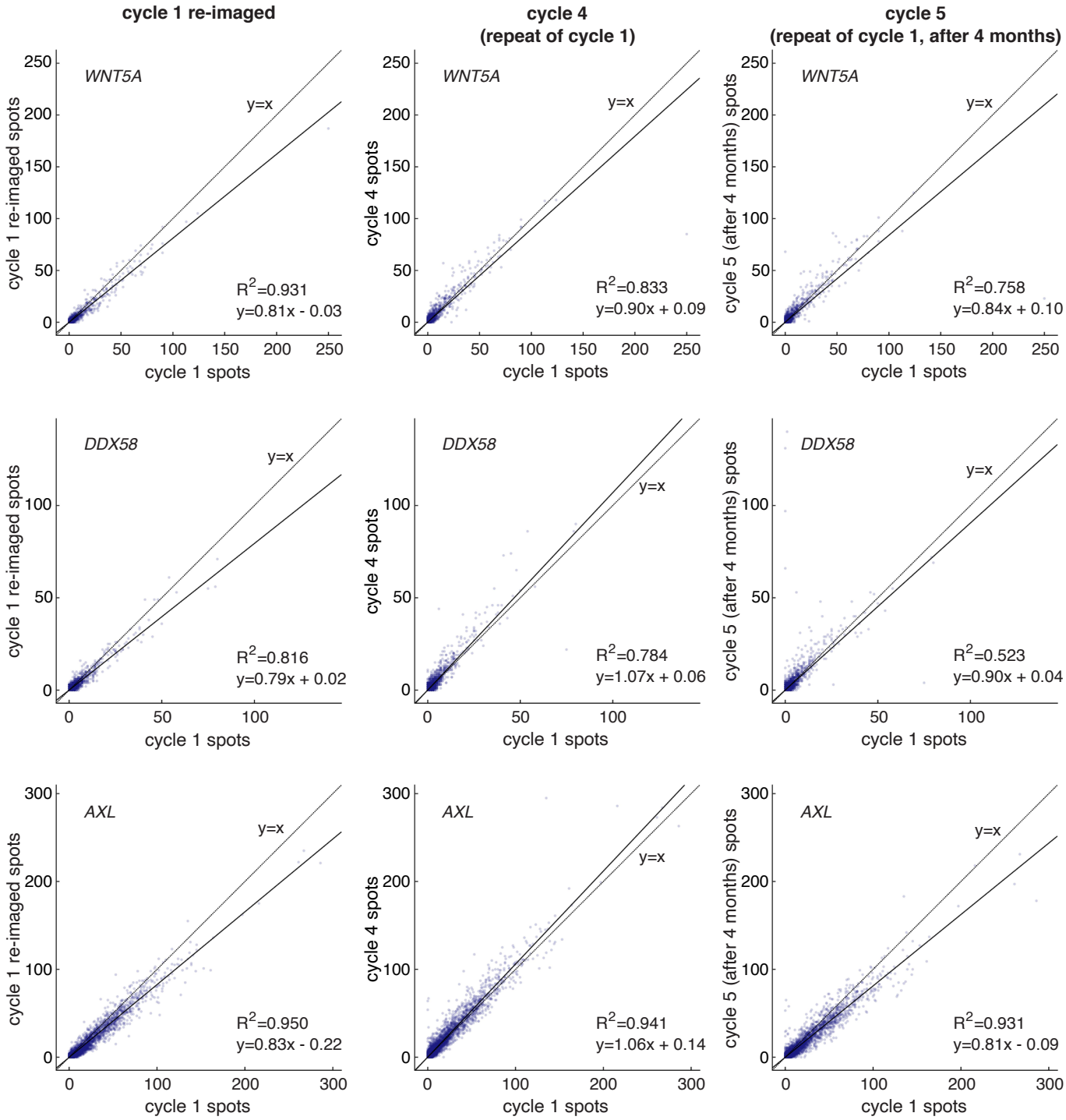
Supplementary Figure 11: Mean fluorescent signal is not well-correlated with spot count density [without pre-readout signal subtraction] and reveals some saturation in spot counts due to optical crowding in rare cells with very high expression levels. Scatter plots of spot count per area of cellular segmentation versus mean background-subtracted fluorescent intensity in the cellular segmentation for 10 different genes probed across readout cycle 1 (left column), readout cycle 2 (middle column), and readout cycle 3 (right column), where scaffolds targeting *UBC* mRNA were probed on every cycle as a positive control. Each dot represents a cell. See Methods section for details.



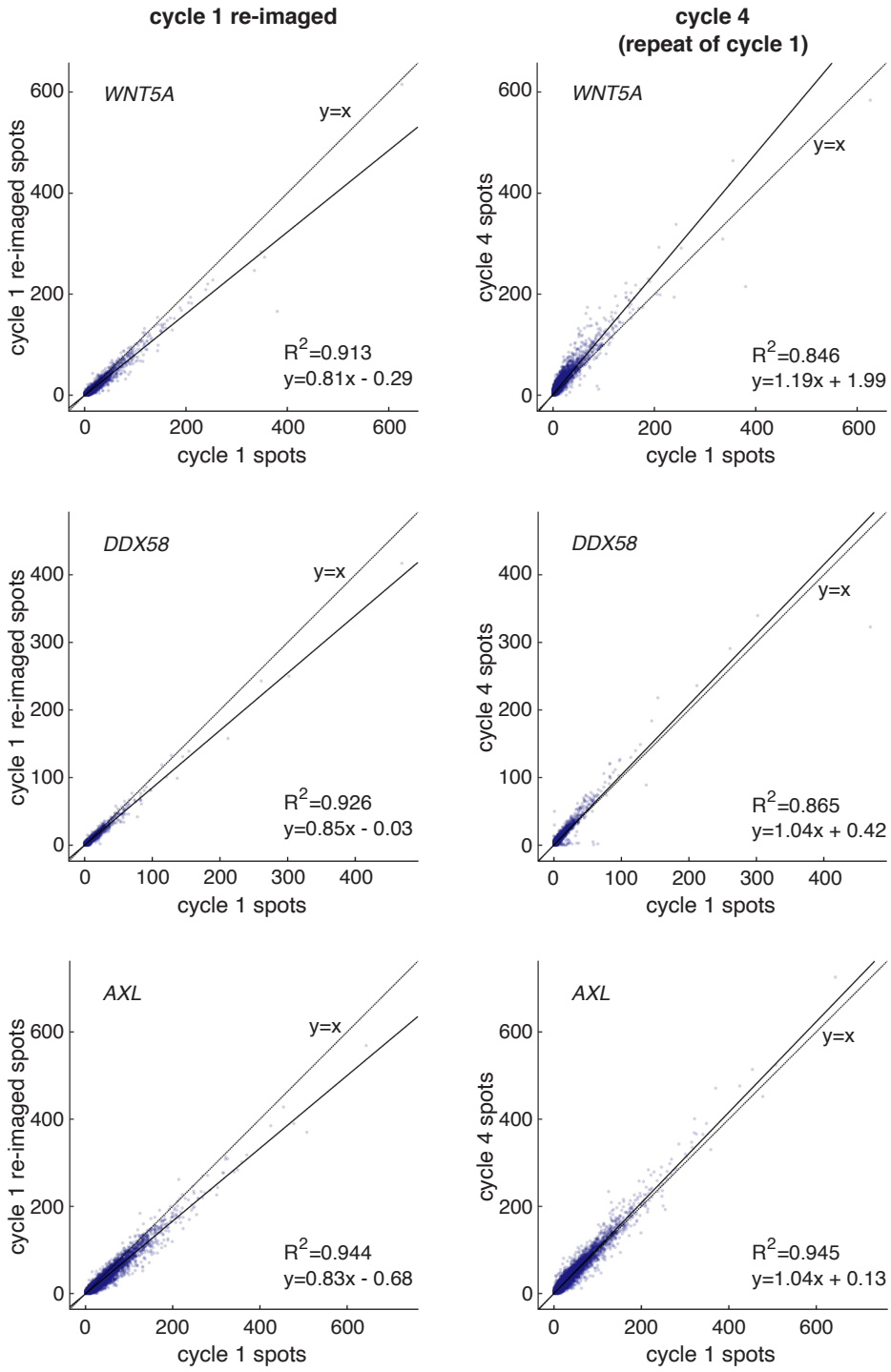
Supplementary Figure 12: Mean fluorescent signal is not well-correlated with spot count density [with pre-readout signal subtraction] and reveals some saturation in spot counts due to optical crowding in rare cells with very high expression levels. Scatter plots, as depicted in Supplementary Figure 11, but with the mean signal before the addition of a given cycle's readout probes subtracted to correct for background from autofluorescence and residual readout probes from previous readout cycles. Data for readout cycle 1 is not available. See Methods section for details.



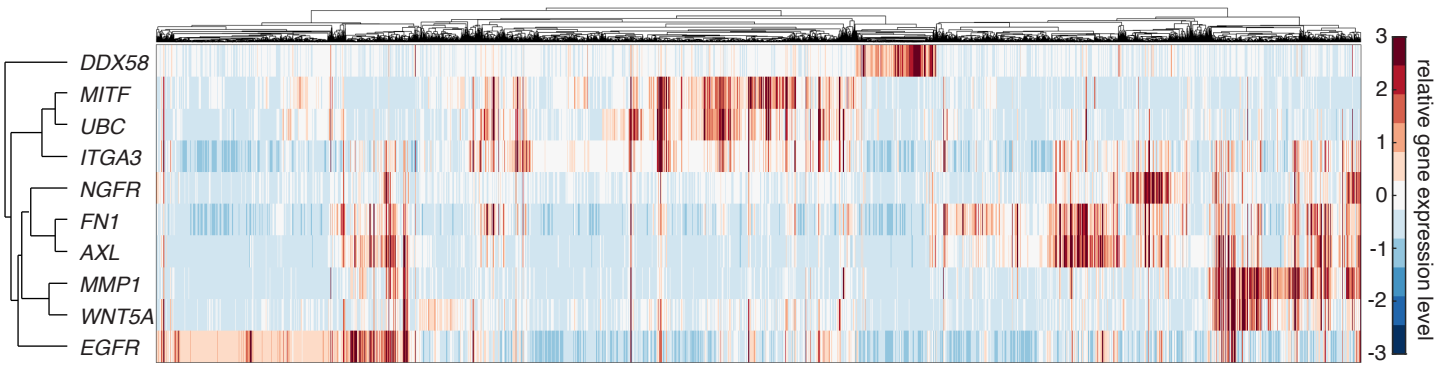
Supplementary Figure 13: clampFISH 2.0 readout probe signal can be removed with a high-stringency wash. (a) Boxplots of clampFISH 2.0 spots per cell detected above a chosen gene-specific threshold for 10 genes before (green) and after (orange) the readout probe stripping protocol. Shown for each gene are spot counts from one of two melanoma lines with higher expression for that gene (for *NGFR*: drug-naive WM989 A6-G3 cells; for all other genes: vemurafenib-resistant WM989 A6-G3 RC4 cells). Each condition contains (from left to right) 39, 39, 39, 48, 48, 48, 44, 44, 44, and 39 segmented cells, where each cell is represented in both the before-stripping and the after-stripping data. For both panels a-b, center lines are median values, bounds of boxes are 25th and 75th percentiles, and whiskers extend to non-outlier minima and maxima, where data falling more than 1.5 times the interquartile range beyond the box bounds are considered outliers. The box and whiskers for the after strip data are at 0 spots and thus are not visible, except for *FN1* which has an interquartile range from 0 to 2.5 spots and a whisker extending to 6 spots. **(b)** Depicting the same data as in (a) for only data below 500 spots per cell.



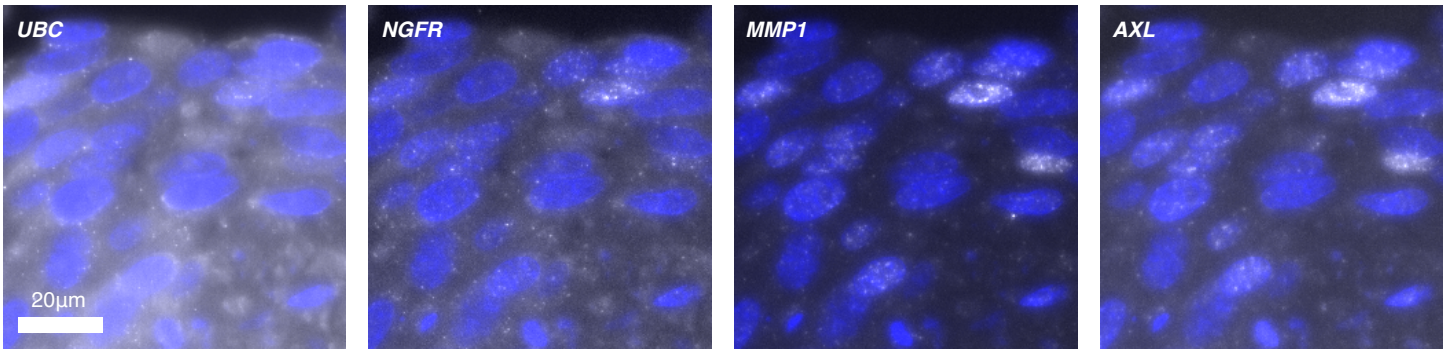
Supplementary Figure 14: clampFISH 2.0 scaffolds remain stably bound after multiple rounds of readout stripping (replicate 1) and storage at 4°C for 4 months. clampFISH 2.0 spots per cell for (from top to bottom) *WNT5A*, *DDX58*, and *AXL* from readout cycle 1 (x-axis) plotted against 3 additional rounds of imaging for the same probed scaffold: re-imaged readout cycle 1 (column 1 plots); readout cycle 4, where the same readout probes were used as cycle 1 (column 2 plots); and readout cycle 5, where again we used the same readout probes as cycle 1 after being stored for 4 months in 4°C (column 3 plots). Each point is one of 44,227 cells. See Extended Data Fig. 9 for experiment workflow schematic. Shown is one of two technical replicates (see Supplementary Fig. 15 for replicate 2).



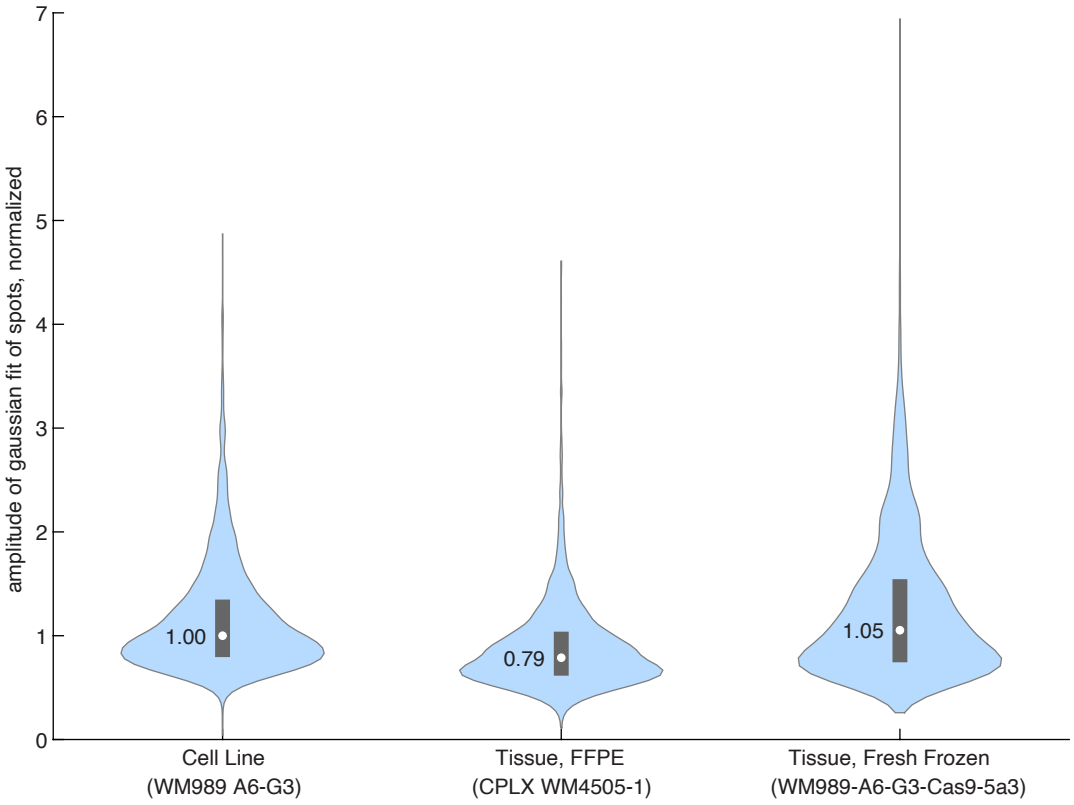
Supplementary Figure 15: clampFISH 2.0 scaffolds remain stably bound after multiple rounds of readout stripping (replicate 2). Technical replicate 2 of the experiment from Supplementary Fig. 14, but without readout cycle 5. clampFISH 2.0 spots per cell for (from top to bottom) *WNT5A*, *DDX58*, and *AXL* from readout cycle 1 (x-axis) plotted against 2 additional rounds of imaging for the same probed scaffold: re-imaged readout cycle 1 (column 1 plots); and readout cycle 4, where the same readout probes were used as cycle 1 (column 2 plots). Each spot is one of 89,545 cells. See Extended Data Fig. 9 for experiment workflow schematic.



Supplementary Figure 16: Clustering of cells expressing one or more drug resistance markers. Technical replicate 2 of the high-throughput profiling experiment from Fig. 3c. clampFISH 2.0 was performed for 10 genes in 253,662 drug-naive WM989 A6-G3 cells. We detected 24,685 cells (10.5% of the 234,410 cells passing quality control checks) that had high levels of one or more of 8 cancer marker genes (*WNT5A*, *DDX58*, *AXL*, *NGFR*, *FN1*, *EGFR*, *ITGA3*, *MMP1*) and performed hierarchical clustering on this population. See methods section for cutoff values defining high expression levels.



Supplementary Figure 17: clampFISH 2.0 in FFPE tissue. We performed clampFISH 2.0 for ten genes in formalin-fixed paraffin embedded (FFPE) tumor tissue derived from human WM4505-1 cells injected into a mouse. We then hybridized readout probes for four clampFISH 2.0 scaffolds, from left to right: *UBC* (Atto 488), *NGFR* (Cy3), *MMP1* (Alexa Fluor 594), and *AXL* (Atto 647N). Shown are images that were taken at 20X magnification. The experiment was performed twice with similar results.



Supplementary Figure 18: Spot intensities of clampFISH 2.0 in a fresh frozen tissue sections were comparable to those in a cell line, while those from a formalin-fixed paraffin embedded (FFPE) tissue section were dimmer. Gaussian-fitted spot amplitudes of clampFISH 2.0 targeting *ITGA3* to round 8 in a cell line (human WM989 A6-G3 cells; left), an FFPE tissue section (human WM4505-1 cells implanted into a mouse; middle), and a fresh frozen tissue section (human WM989-A6-G3-Cas9-5a3 cells injected into a mouse), normalized to the median amplitude of the cell line spots. The clampFISH 2.0 primary and amplification steps were performed in parallel for all sample types, which were all imaged on the same microscope at 20X magnification with equivalent (1 second) exposure times. Numbers shown are median normalized amplitudes. Circles and numbers shown are median values and bounds of boxes are 25th and 75th percentiles. n= 3234, 2973, and 1669 spots (from left to right).