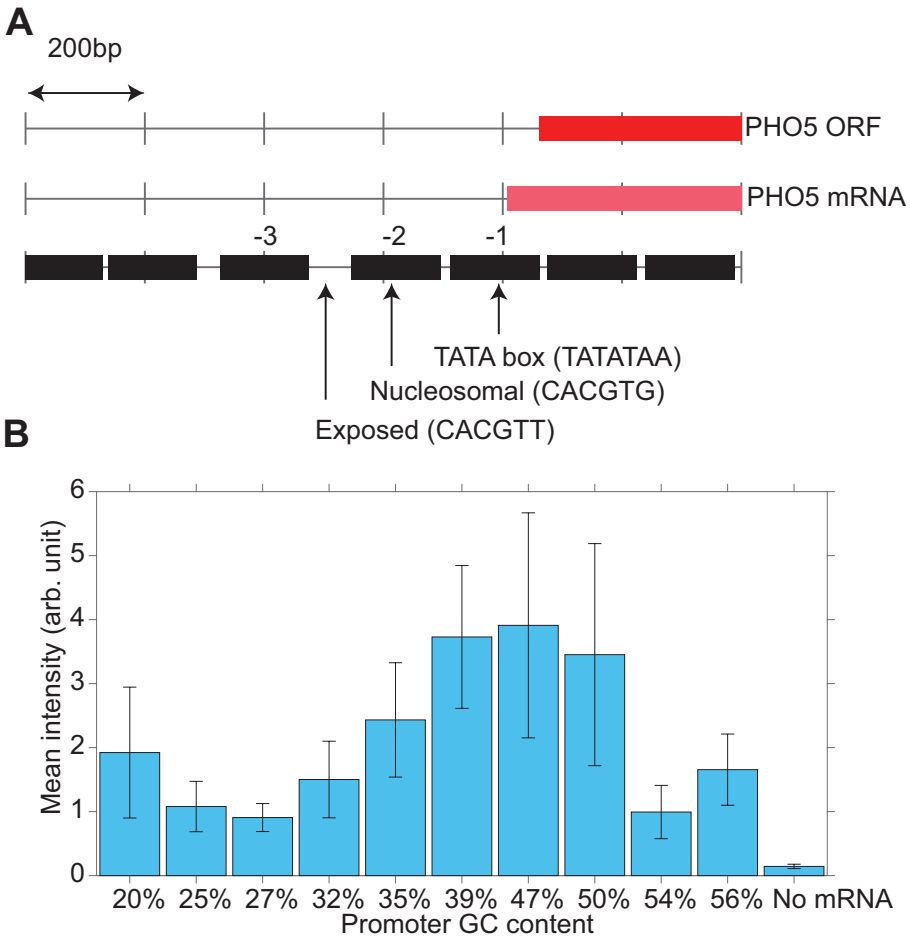
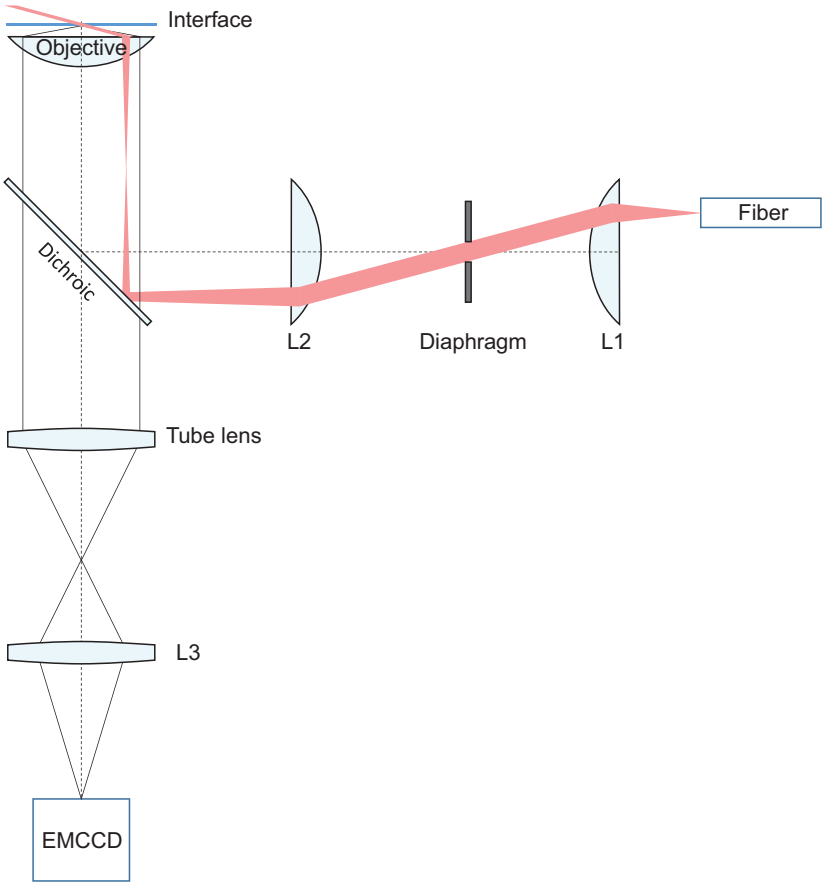
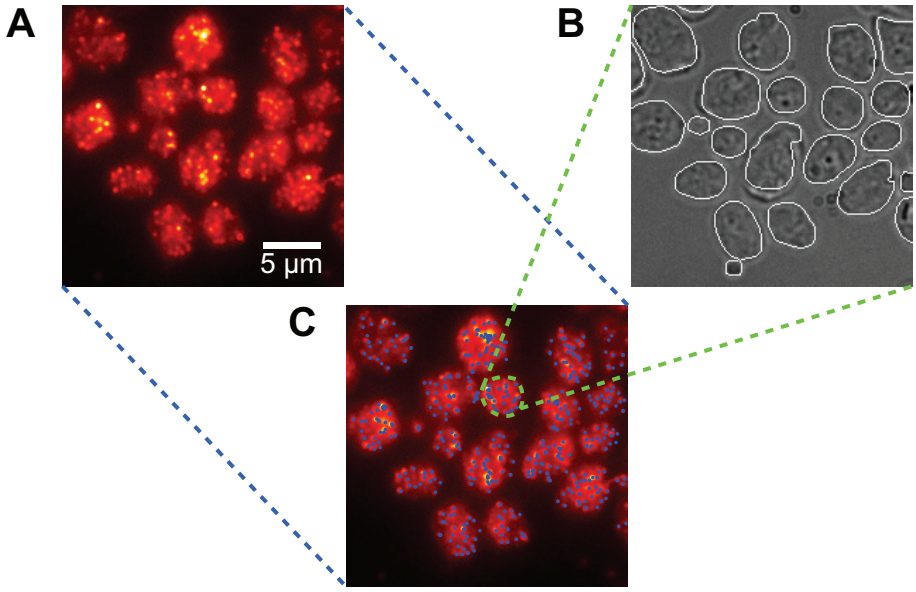


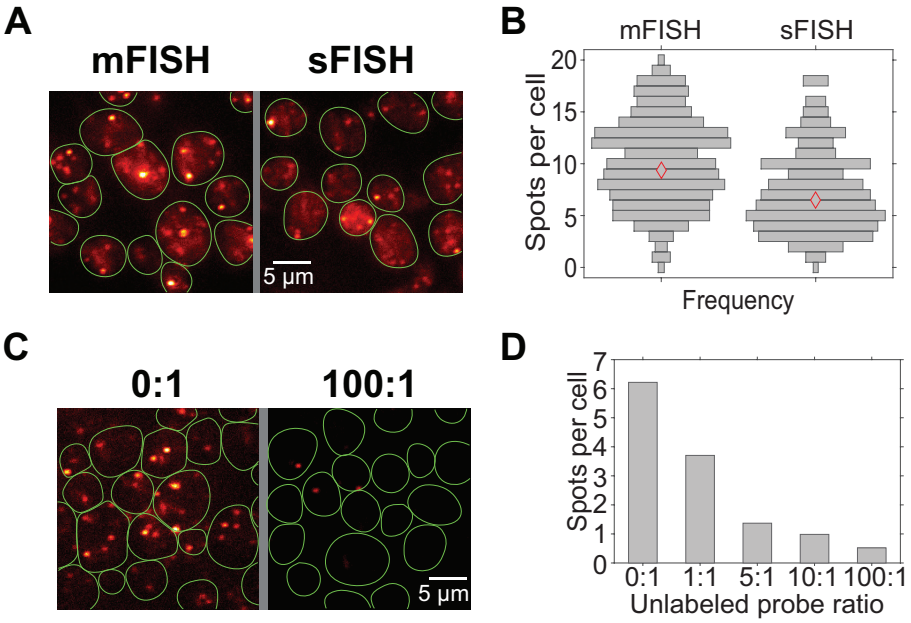
Supplementary Figure S1: PHO5 promoter variants used in this study. (A) PHO5 promoter map. The open reading frame (ORF) and the transcribed region of the PHO5 gene are shown. The reference nucleosome map is retrieved from [1]. The first three nucleosomes are numbered from -1 through -3. The wild-type PHO5 promoter contains two Pho4 binding sites, one in the exposed region between nucleosome -3 and -2, and one within nucleosome -2. The nucleosomal site (CACGTG) has a stronger affinity to Pho4 than the exposed site (CACGTT). The promoter variants used in this study have a common lone high affinity site (CACGTG) in the exposed region with various GC% sequences in the nucleosome -2 region. (B) YFP Expression Level. YFP intensity is plotted against the percentage of GC in the promoter sequence of the strain. This is a non-monotonic relationship that shows the lowest expression in the 27%GC strain and the highest in the 47%GC strain. Error bars show the standard deviation of the population which is much larger for strains that are more highly expressing.



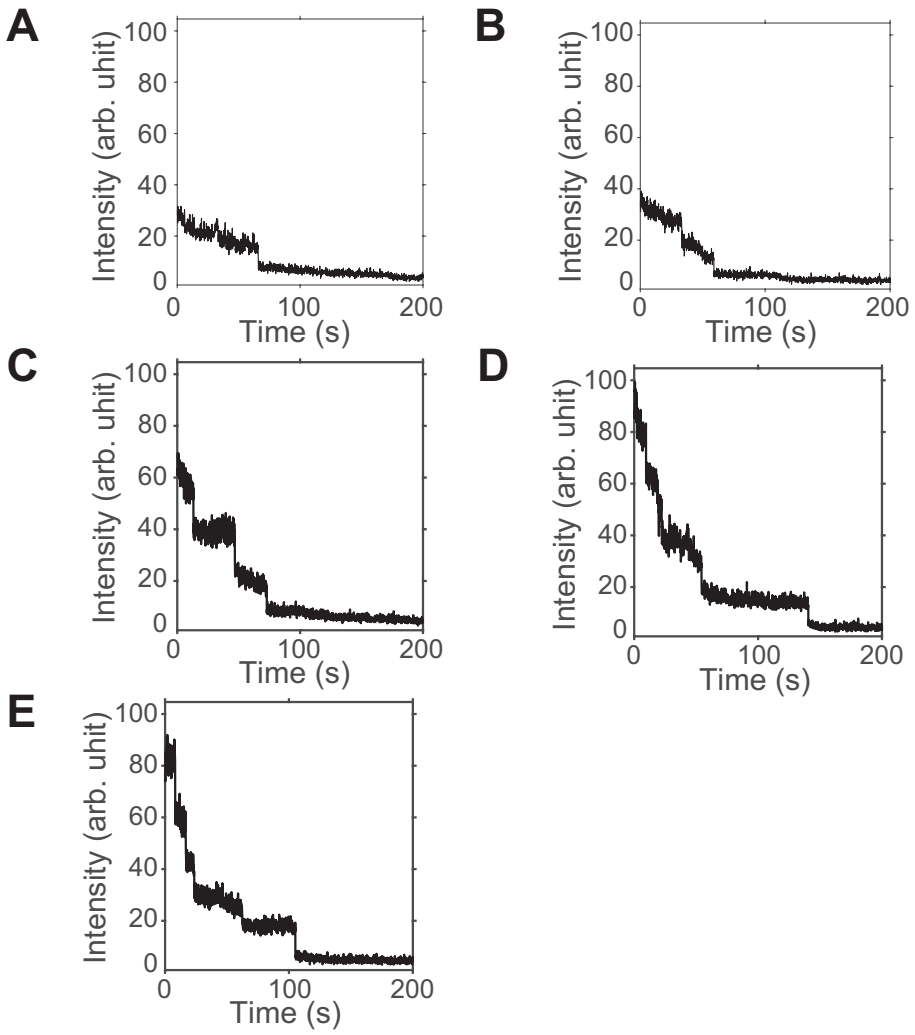
Supplementary Figure S2: Optical setup for fluorescence microscopy. A laser line is selected by the AOTF (not shown) and coupled to the fiber optic. The fiber output can be laterally translated with respect to lens L1 to vary the incidence angle. L1 collimates the output beam from the fiber, and lens L2 focuses the beam on the back focal plane of the objective. The illumination area is adjusted using the diaphragm located at the image conjugate plane between L1 and L2. The image formed by the tube lens (f=18 cm, Olympus) outside the microscope is relayed onto the EMCCD by lens L3 with some magnification.



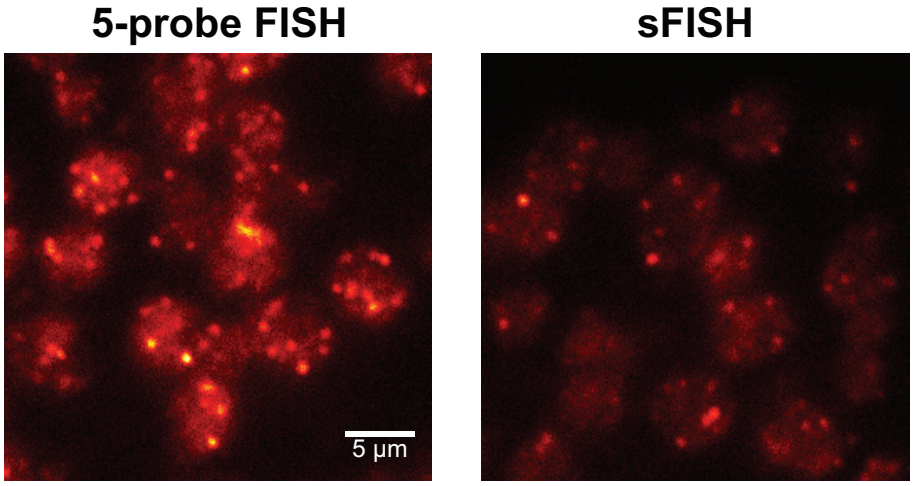
Supplementary Figure S3: Image processing. (A) Raw sFISH data from the Cy5 channel for an mRNA expressing strain. (B) Detection of cell boundaries by applying Sobel filter on the DIC image stack. The local background is approximated by averaging the pixels near the boundary. (C) Spot detection. All local maximum intensity pixels are considered candidate spots. The distribution of the background-subtracted spot intensities exhibits a peak near zero and another peak centered at a higher intensity. Only the spots that belong to the higher intensity peak are qualified as true spots.



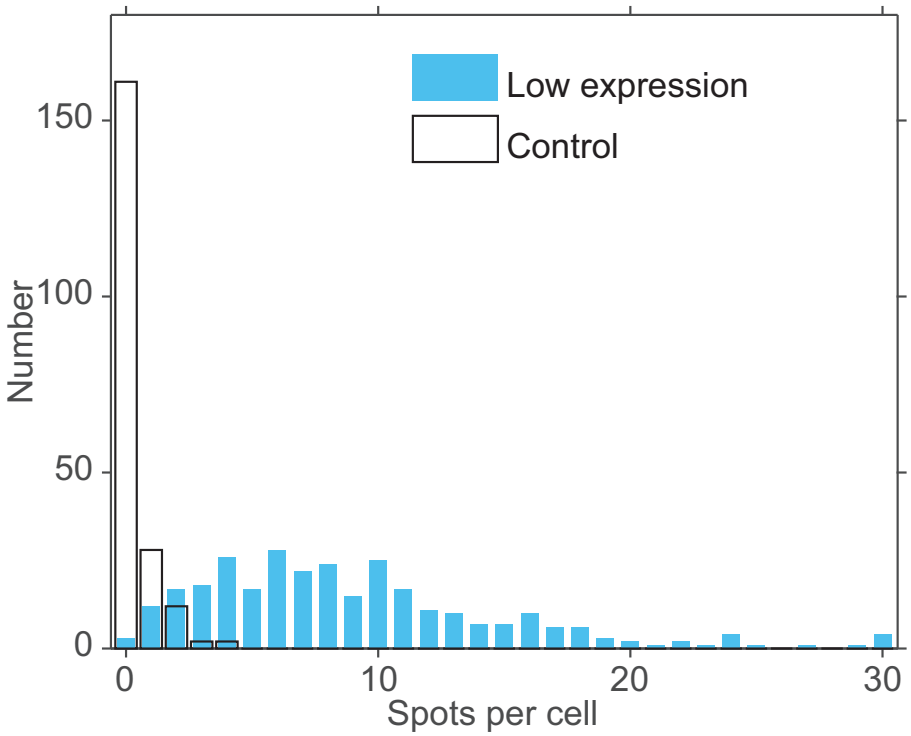
Supplementary Figure S4: Control experiments with KAP104 FISH. **(A)** Comparison between mFISH and sFISH. mFISH and sFISH targeting the KAP104 transcript were performed on the cells grown in the same tube, but fixed with either formaldehyde (mFISH) or methanol (sFISH). Cell boundaries are shown in green. In both cases punctate spots can be seen. mFISH spots are only 2 to 3 times brighter than sFISH spots despite using as many as 48 probes. The relatively low fluorescence signal of mFISH spots compared to sFISH spots is due to the dye (Quasar 670 vs. Cy5) and illumination geometry (epi vs. inclined). **(B)** Spot count distributions from mFISH and sFISH. In these horizontal bar graphs, the position on the vertical axis represents the number of spots per cell, and bar width represents the frequency. Red diamonds are the mean values (9.4 for mFISH and 6.5 for sFISH). The wide distributions represent cell-to-cell variability, not experimental noise. **(C)** sFISH images with (right) and without (left) an unlabeled competitor probe. The ratio above each image is the ratio of unlabeled to labeled probes. **(D)** Mean number of spots per cell vs. unlabeled probe. The spot count decreases monotonically with the concentration of unlabeled probes. The concentration of labeled probes is fixed in the protocol. At 100:1 ratio, only $\sim$0.5 spots per cell are seen, which is similar to the rate of false positives.



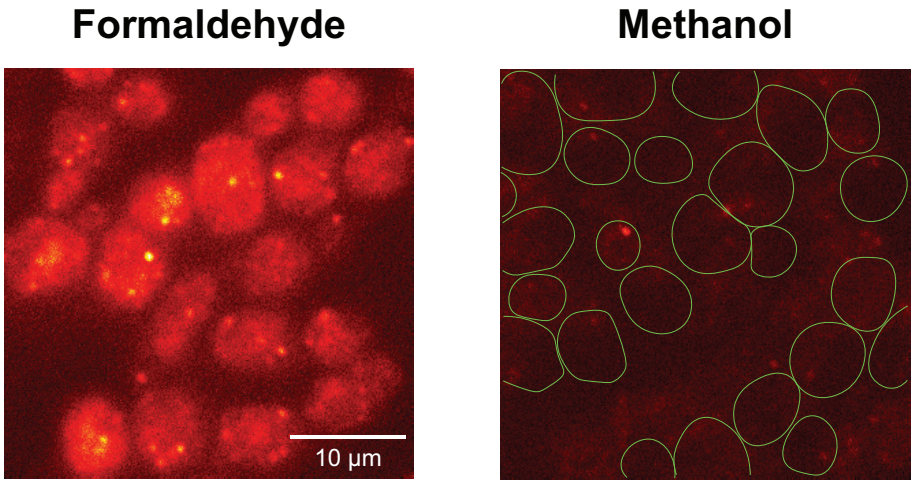
Supplementary Figure S5: Photobleaching of FISH spots. Fluorescence intensities from single spots were monitored under continuous excitation. Most sFISH spots show photobleaching in a single step, (A), which is evidence for the presence of a single fluorophore. Subsequent panels from (B) to (E) show traces taken from 2, 3, 4, and 5 probe treatments, respectively. Overall, the number of photobleaching steps increases with the number of probes used. For these acquisitions, the exposure time was set to 100 ms.



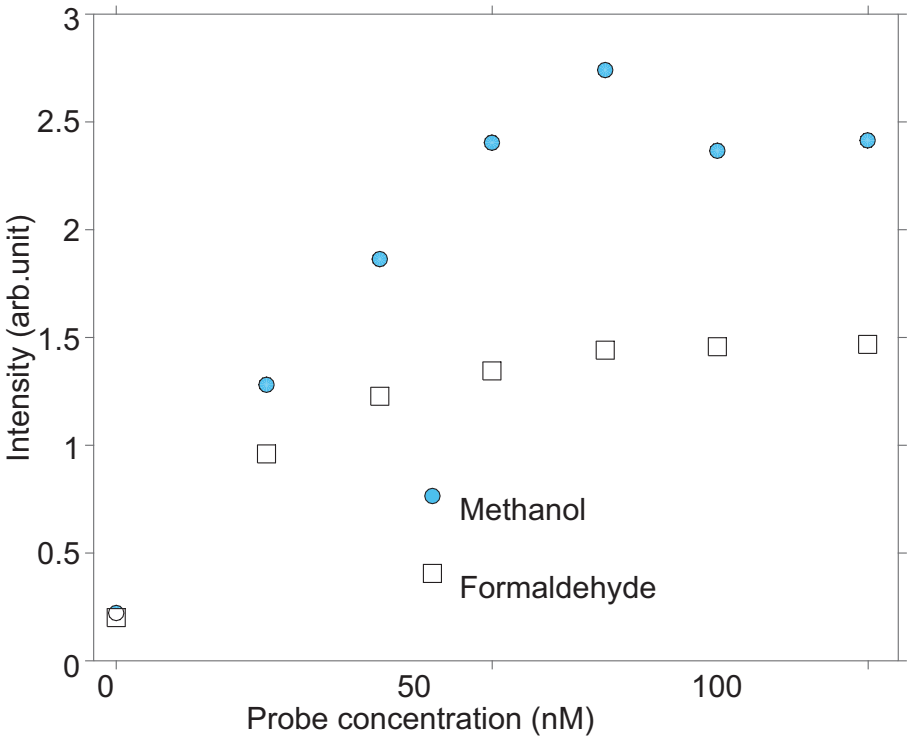
Supplementary Figure S6: Comparison of FISH with five probes and a single probe. Raw FISH images for the low expression yEVenus strain are shown for five Cy5-labeled probes (left) and a single Cy5-labeled probe (right). The average intensity of spots is about three-fold higher when five probes are used.



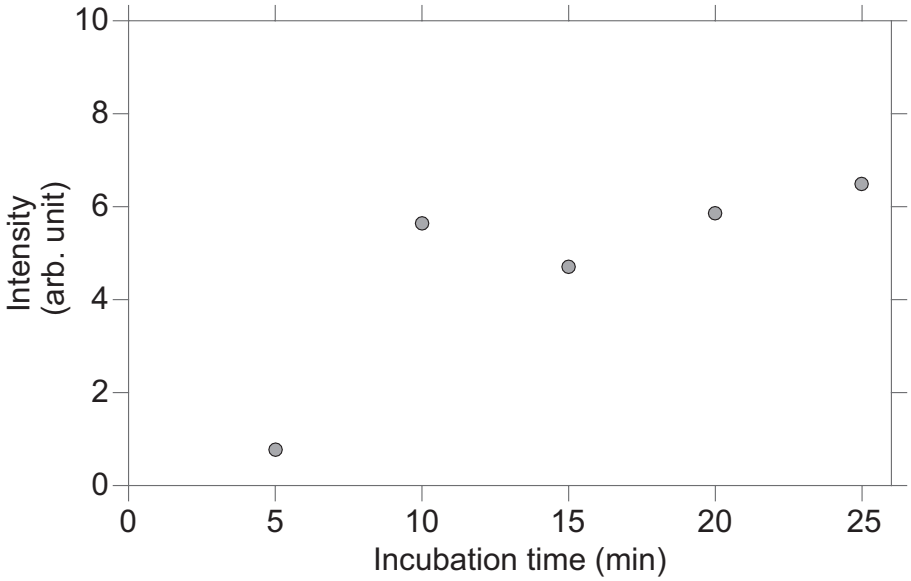
Supplementary Figure S7: False positives in sFISH. Spots counted in each cell are plotted as a histogram. The negative control strain yields a false positive rate of less than 1 per cell (transparent bars). For comparison, the distribution from the low expression strain (positive control) is shown in blue bars.



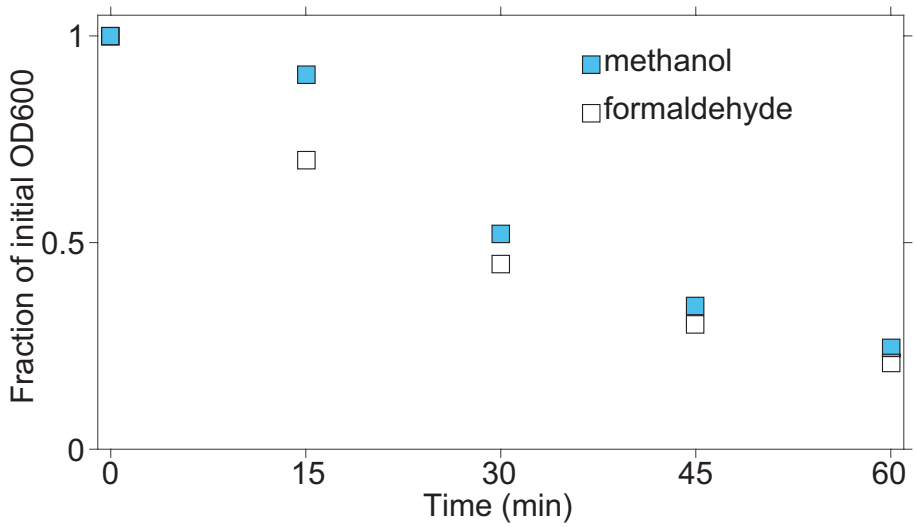
Supplementary Figure S8: Formaldehyde vs. methanol. Negative control cells treated with single probes are shown with formaldehyde fixation (left) and methanol fixation (right). Formaldehyde-fixed cells exhibit higher cellular background as well as more punctate spots (false positives) than methanol-fixed cells. Cell boundaries are shown only for methanol-treated cells.



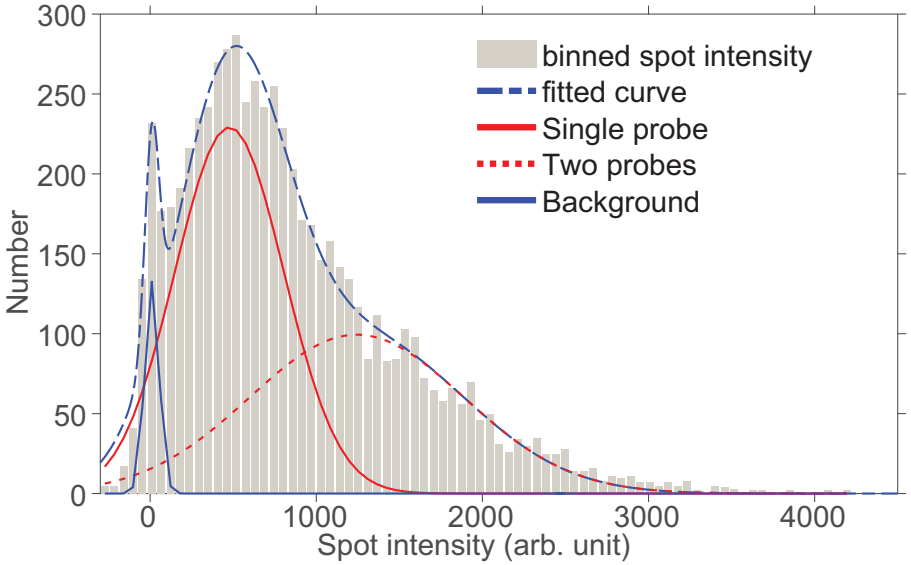
Supplementary Figure S9: sFISH signal vs. probe concentration. sFISH was performed on both methanol- and formaldehyde-fixed cells over a range of probe concentrations. The plot shows that the average fluorescence signal per cell increases with probe concentration and plateaus around 60 nM. Each data point is an average from 200-300 cells. Based on this relationship, the probe concentration of 65 nM is selected for the standard sFISH protocol.



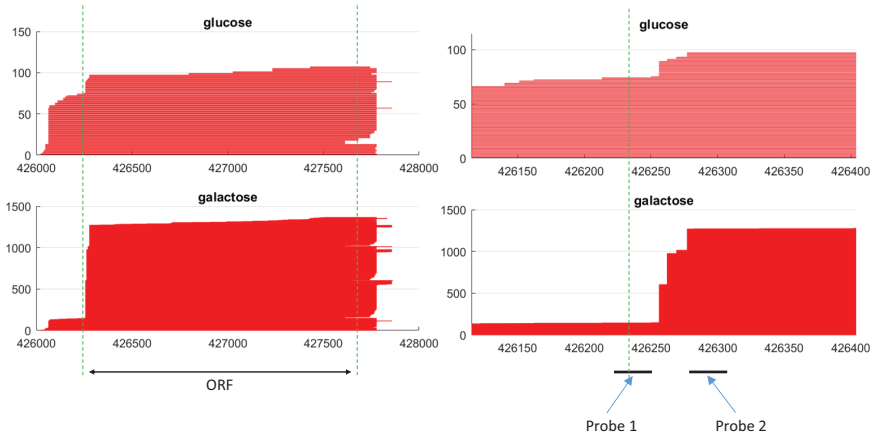
Supplementary Figure S10: sFISH signal vs. zymolyase incubation time. sFISH was performed on cells spheroplasted in zymolyase for different amounts of time. The subsequent probe treatments were identical. As shown in the plot, the average fluorescence intensity per cell plateaus at 10 minutes of incubation. Each data point is an average from 200-300 cells.



Supplementary Figure S11: Spheroplasting by zymolyase confirmed by absorbance measurement (OD600).



Supplementary Figure S12: Spot ambiguity. Spots inside a cell are not uniformly bright. Using a single fluorophore requires every spot to be considered equally. By integrating the area under the highest intensity Gaussian we can say how often an ambiguous spot occurs. In our lowest expressing cell we find on average one ambiguous spot. We can say that the fit represents background peaks, single fluorophores and ambiguous spots. We find that the rate of ambiguous spots in our highest expressing strain is about 4.4 per cell (13% of spots.)



Supplementary Figure S13: Determination of probe location for mRNA isoform profiling. mRNA isoform data for a yeast gene RGL1 (YPL066W) in glucose (top row) and galactose (bottom row) are shown at different zoom levels (zoom-out view on the left column and zoom-in view on the right). The x-axis represents the genomic coordinates around RGL1 on Chromosome XIV. Green vertical lines mark the ORF boundary. mRNA isoforms published in Pelechano et al. [2] are represented by red horizontal lines stacked vertically in the order of start coordinate. As shown, the transcriptional profile of RGL1 changes dramatically from glucose (top row) to galactose (bottom row). The target locations of Probe 1 and Probe 2 are shown as black bars.

Supplementary Table S1: DNA sequence in the region of nucleosome -2.

GC%	Sequence
20	AAAGTTAATTTAATATGCAATTTCTAAATTAATCGATACAACCTTAACAATAAAAGCTTGAATTAGCAAAGACTAAATTTATTATTATTGTACATTTTTTCAAATAGATTGCACATTCAAAATTATCAAATTGGTTACATTATTTTA
25	ATAGTTAATTAAATAGTAAATCTTTAAATGAATAGATACAACCTTGGCACTCAAAGCTTGGAATATTACAGACTAAATTTATAATTATATTACTTGTTTTCGAAAAGATCGCAAATGTCAAATTATCAAATTGTTCAACTTACTTGG
27	AGAGTTAATTTAATATGCAATATCTAAATTAATCGATAAAACCTTTGCAATAAAAGCTTGGACTAACATAGACTAAATTTATGATTCTATTCTCTGTTTTCAAAGAGATCGCAAATGCAAAATTATCAAATTGGTCACCTTACTTGT
32	AGAATTAATTGAATAGACAATCTCTAAATTAATCGATACAACCTTGGCACTCAAAGCTTGGACTAGAACATACTAAATTTATGATTCTTGTTTCTGTTTTCGAAGAGATCGCAAATGCCAAATTATCAAATTGGTCACCTTACTTAG
35	AGAGTTAATTGAATAGGCAATCTCTAAATGAATATATACAACCTTGGCACTCAAAGCTTGGACTAGCACAGACTAAATTTATGATTCTGGTCCTTGTTTTCGAAGAAATCGCAAATGCCAAATTATCAAATTGGTCACCTTACTTGT
39	AGAGTTAATTGAATAGGCAATCTCTAAATGAATCGATACAACCTTGGCACTCAAAGCTTGGACTAGCACAGACTAAATTTATGATTCTGGTCCCTGTTTTCGAAGAGATCGCACATGCCAAATTATCAAATTGGTCACCTTACTTGG
47	AGAGGTAATTGAACAGGCGATCTCTAAATGAATCGACACCACCTTGGCACTCAAAGCTTGGGCTAGCACAGACTAAACTTGTGATTCTGGTCCCTGGTTTCGAAGAGATCGCACATGCCAAACTATCAAGTTGGTCACCTTACTCGG
50	GGAGCCAATTGAATAGGCAATCTCTGAATGAATCGGTACAACCTTGGCACTCAAAGCTTGGGCTAGCCCAGACTCCATGTATGATTCGGGTCCCTGTTGTCGACGAGATCGCACATGCCAAATTACCAAATTGGCCACCTGACTGGG
54	AGAGTGAATTGAGTAGGCAGCCTCTAAACGCATCGACACAGCCGTGGCACTCAAAGCTTGGACTAGCGCAGCCTGAACGTACGATTCTGGTCCCCGTTTTCGAAGCGATCGCGCGTGCCAAGTCATCACATTGGTCACCTTACTTGG
56	AGGGTCCATTGGGCAGGCAAGCCCGAAAGGAACCGATACAACCGTGGCACTCAAAGCTTGGACTAGCCCAGGCTACATCGATGATTCGGGTCCCTGTTGTCGAAGAGCTCGCGCATGCCAAACCATCACATTGGTCGCCTGACTTGG

Supplementary Table S2: DNA sequence for yEVenus probes of similar melting temperature. Probes were designed to have similar GC content and melting temperature. The first probe is used for all of the single probe experiments.

Melting Temp($^\circ C$)	Sequence
47.7	CA/iCy5/ACCAAAATTGGGACAACACCAGTG/3BioTEG/
49.7	TT/iCy5/ACCGTAAGTAGCATCACC
51.1	TG/iCy5/ACTTTTTCAAGTCTGCCATG
47.8	AC/iCy5/CGGAGACAGAAAATTTGTGACC
49.2	CC/iCy5/ATGGAACTGGCAATTTACC

Supplementary Table S3: Probe sequences. Listed are sequences of probes used against yEVenus mRNA sequence[3]. These sequences are identical to all but two used in a previous study[4].

Probe Number	Sequence
1	AATTCTTCACCTTTAGACAT
2	AATTGGGACAACACCAGTGA
3	CATCACCATCTAATTCAACC
4	GACAGAAAATTTGTGACCAT
5	CATCACCTTCACCTTCACCG
6	AAGGTCAATTTACCGTAAGT
7	GTTGGCCATGGAACTGGCAA
8	ATAACCTAAAGTAGTGACTA
9	TGTTGTTTCATATGATCTGG
10	CATGGCAGACTTGAAAAAGT
11	CTTTCTTGAACATAACCTTC
12	GTCATCTTTGAAAAAAATAG
13	CAGCTCTGGTCTTGTAGTTA
14	GTATCACCTTCAAACTTGAC
15	TAATTCGATTCTATTAACTA
16	TCTTCTTTAAAATCAATACC
17	TTTGTGACCTAAAATGTTAC
18	GAGAGTTATAGTTGTATTCC
19	TCAGCAGTGATGTAAACATT
20	CTTTGATACCATTCTTTTGT
21	TTGTGTCTAATTTTGAAGTT
22	TTGAACACCACCATCTTCAA
23	TTTGTTGATAATGGTCAGCT
24	GGACCATCACCAATTGGAGT
25	AATGGTTGTCTGGTAACAAG
26	AAGGCAGATTGATAGGATAA
27	CTTTTCGTTTGGATCTTTGG
28	CTAACAAGACCATGTGGTCT
29	ATACCAGCAGCAGTAACAAA
30	ACAATTCATCCATACCATGG

Supplementary Table S4: Probe sequences. Listed are sequences of probes used against KAP104 mRNA sequence. These sequences are identical to those used in a previous study[5].

Probe Number	Sequence
1	GAGTTGCTAGTTGCAACACA
2	TAGCTTCCATTGCGTTATTA
3	TCTGAAGATCCTGTAGGGAG
4	ATTAAATTGTTTCCCCCTAG
5	TACCTGTCACGTTCGAAACG
6	CTCTATGCTGCCTATAGTAA
7	GCATTTGAAGACCAGTTGGA
8	ATTTGAGGTTAGCTCTAGCA
9	AAGCCTTAATACTTGGCTCA
10	AAAATTGAGCGCTGTCTTCC
11	CTATCCAATAAGGCTTCCAT
12	CTGAGCGAATCACAGGTGAG
13	AACTTTGTGTTTGTAGCGGG
14	TAATGCAAATCTGTGCCCTA
15	AAACCAGCTTATCTGGTCTG
16	TTTCCTCATTTACAGTGGTG
17	TGCAAAAACTCGCAGGCTTC
18	GTTCAGGAATATTTGGGCTC
19	GAAGCTTCCAGGAGAACGAT
20	CTTTATCCTCCAAGAATGCA
21	TTTTTCACAATACGGGGTGC
22	TCGTCATTACATCTAGCGTT
23	ACGCTATATCCATCACTTGA
24	GCTTCTCTAATAAACCACCT
25	AATACTTCATTCCACCTTCT
26	TGGTATTAGTGCTGGTAAGC
27	ACATGTCATTTTCCTCACTG
28	GGAGTCGGCGTTTTCAATAA
29	GGGCGGCAAAATTATTTGCA
30	TCACTGTCTGACAGCAAAGC
31	AAGGCTCTGTTGTACACTTC
32	TTCTCAGGCACTACTATTGT
33	TCCCTTGAGGGAACAATAGA
34	TCCTGCAAACATTCTAGCAT
35	GCAAAGCAGCTTTGTCTTAC
36	TTCCGTACCAATCAACTTCA
37	ATCACTATCGTCGTTATGCA
38	ATCGCATTTATCACAGCAGG
39	ATCACAGAGCTGTCTACGAT
40	TTCCGATGGTCACAGACAAG
41	GGAATCATTGGCAAATGCGC
42	GCGTTAACGGACAAACACCA
43	CTACTCTTTTCCTCTACATC
44	CCGTGCTGGTCAAATTGATA
45	TCTACATTGCTTGAAAGGCC
46	GTGTAGATCTCTTGCGCAAA
47	TTTGGGCAGAATGGTTCATC
48	AGAAGGAGATTTCATCGGGC

Supplementary Table S5: DNA sequence for KAP104 probes used in sFISH experiments.

Melting Temp($^\circ C$)	Sequence
56.0	CA/iCy5/CAGGTGAGAAATTTGGATTCGA
55.4	CACAGGTGAGAAATTTGGATTCGA

Supplementary Table S6: DNA sequence for RGL1 probes.

	Melting Temp($^\circ C$)	Sequence
Probe 1	60.2	[Cy5]TATAACTGGGTGCGTCATTTTCACTTCTTCG
Probe 2	68.3	[Cy5]TAACGTATCAGGTAAACCGGGGCAGCCCCGT

References

[1] Jiang, C. and Pugh, B. F. (2009) A compiled and systematic reference map of nucleosome positions across the Saccharomyces cerevisiae genome. *Genome Biol,* **10**(10), R109.

[2] Pelechano, V., Wei, W., and Steinmetz, L. M. (2013) Extensive transcriptional heterogeneity revealed by isoform profiling. *Nature,* **497**(7447), 127.

[3] Sheff, M. A. and Thorn, K. S. (2004) Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae. *Yeast,* **21**(8), 661–670.

[4] To, T.-L. and Maheshri, N. (2010) Noise can induce bimodality in positive transcriptional feedback loops without bistability. *Science,* **327**(5969), 1142–1145.

[5] Dodson, A. E. and Rine, J. (2015) Heritable capture of heterochromatin dynamics in Saccharomyces cerevisiae. *Elife,* **4**, e05007.