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Single cell systems biology by super-resolution imaging and combinatorial labeling

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Abstract

Fluorescence microscopy is a powerful quantitative tool for exploring regulatory networks in single cells. However, the number of molecular species that can be measured simultaneously is limited by the spectral separability of fluorophores. Here we demonstrate a simple but general strategy to drastically increase the capacity for multiplex detection of molecules in single cells by using optical super-resolution microscopy (SRM) and combinatorial labeling. As a proof of principle, we labeled mRNAs with unique combinations of fluorophores using Fluorescence *in situ* Hybridization (FISH), and resolved the sequences and combinations of fluorophores with SRM. We measured the mRNA levels of 32 genes simultaneously in single *S. cerevisiae* cells. These experiments demonstrate that combinatorial labeling and super-resolution imaging of single cells provides a natural approach to bring systems biology into single cells.

Introduction

Systems biology seeks to quantitatively understand the interactions between many biological components. To do so two distinct technical approaches are employed: genomics^{1–4}, which permits the analysis of all genes and proteins in a system simultaneously, and single cell biology^{5–10}, which follows the behavior of genes in individual cells within their native spatial context. However, these approaches have complementary limitations: genomics averages over heterogeneity and spatial complexity within populations, while single-cell methods have been limited to the study of a few genes at a time.

To unify the two approaches, we propose to use super-resolution microscopy to bring genomics into single cells. SRM^{11–14} has been a powerful tool for cell biology and can resolve sub-cellular structures down to 10–20 nm. This extraordinary resolution can be harnessed for systems biology: for the typical yeast cell of 100 μm^3 , the 10–20 nm resolution

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Author Contributions

E.L. and L.C. performed the experiments, carried out the analysis, and wrote the manuscript. L.C. conceived the idea and designed the experiments.

The authors declare there are no competing financial interest.

of SRM translates into approximately 10^8 independent volume elements (voxels) per cell. These voxels provide enough space to detect large numbers of fluorophore-based barcodes attached to molecular species. Using only 9 of the photoswitchable fluorophore pairs currently available for SRM, over a hundred combinations of quadruplet ($9C_4=126$) barcodes can be used. The abundances of each molecule can be quantified by counting the number of times the corresponding barcode is observed in the super-resolution image of the cell, within their native cellular and inter-cellular contexts.

To demonstrate the feasibility of this approach, we performed proof-of-principle experiments to detect multiple mRNA species in single *Saccharomyces cerevisiae* cells. We based our approach on the single molecule FISH (smFISH) technique^{15,16}. We used smFISH to combinatorially barcode transcripts, taking advantage of the high labeling specificity of oligo probes. We employed two different barcoding strategies, spatial and spectral. The first relied on the spatial ordering of fluorophores from many small oligonucleotide probes bound to the transcripts. The second relied only on the combination of colors used in the probes. Although the first strategy allows higher multiplexing, the second strategy is less technically demanding and more robust. We therefore used spectral barcoding to profile transcripts from 32 stress responsive genes in single *S. cerevisiae* cells to demonstrate the method. In comparison to previous multiplex FISH approaches that labeled chromosomal loci and transcriptional active sites¹⁷⁻¹⁸ our approach directly barcoded single mRNAs.

Results

Spatial coding of single mRNAs

The first strategy we explored for combinatorial labeling directly resolved the spatial ordering of different fluorescent oligonucleotide probes on individual mRNAs. As 20mer probes are approximately 7 nm in length, regions of the mRNA separated by 100 nucleotides and hybridized as a group of 4-5 probes can in principle be resolved by SRM with its 10-20 nm resolution. By labeling the probes with different fluorophores that hybridize to mRNA in a specific pattern, a nanoscopic barcode can be resolved on each transcript (Fig. 1).

We targeted the *PUN1* mRNA in single *S. cerevisiae* cells with 3 sets of oligo probes labeled with different fluorophores. These probes were tiled along the mRNA in a 5' to 3' spatially ordered fashion. Hybridized mRNAs appeared as co-localized and diffraction-limited spots (Fig. 1a). We observed that $96 \pm 2\%$ ($n=29$ molecules, s.e.m.) of spots co-localized in all three channels, indicating efficient hybridization of the *PUN1* probes. We quantified the hybridization efficiency of a single FISH probe to be $H_{single} = 67.5 \pm 9.1\%$ by photobleaching (Supplementary Fig.1, $n=173$, s.e.m.). The effective hybridization efficiency of the barcode, or the probability that at least one probe is hybridized for each coding position is $H_{barcode} = (1 - (1 - H_{single})^R)^N$, where R is the probe redundancy at each coding position and N is the number of coding positions. With 4 probes in each code position, the labeling efficiency is 99% for each position giving a 96% coincidence rate for all 3 coding positions.

The position of labeled probes on mRNA can be determined to a much higher resolution by gaussian fitting^{19,20} of the co-localized spots. We observed the correct spatial order in $74\pm 8\%$ ($n=28$, s.e.m.) of 3-color co-localized mRNAs following alignment. Error in detecting the order of the barcode (26%) may result from a combination of factors, including localization error, lack of z resolution, and mRNA secondary structure (Supplementary Note). Barcode readout fidelity was robust to switching the barcode order (Fig. 1e-h), independent of mRNA identity (Supplementary Fig. 2-4)

Super-resolution imaging of mRNA barcodes

Barcoding with conventional fluorescence microscopy is only useful in cases where transcript levels are low. When the density of transcripts is high, diffraction-limited fluorescent spots of the same color will overlap and make barcode readout impossible. Super-resolution imaging using sparse activation of subsets of overlapping fluorophores is essential for high-density multiplex barcoding when signals from multiple transcripts overlap.

To perform super-resolution barcoding, we turned to STORM (Stochastic Optical Reconstruction Microscopy) imaging with Cyanine dye based photoswitchable dye pairs²¹. We used singly-labeled probes that only generated photoswitchable pairs when an emitter-labeled and an activator-labeled oligonucleotide probe hybridized next to one another on the mRNA (Fig. 2a). As both probes are required for the fluorophore to be re-activated, background is dramatically reduced because non-specifically bound emitter probes cannot reactivate. We used the activator-emitter dye pairs to construct our barcodes, with Cy5, Alexa 680 and Alexa 750 as emitters (Supplementary Fig. 5). and Alexa 405, FITC and Cy3 as activators²¹.

We labeled the *PUN1* mRNA (Fig. 2a) using Cy5-based probe pairs. With 4 probe-pair redundancy, we observed a 3 color hybridization rate of $61\pm 8\%$ ($n=85$, s.e.m.), consistent with the theoretical hybridization efficiency of $H_{\text{superbar}}=75\%$ (Supplementary Note and Fig. 1). Hybridization efficiency can be improved by using a higher redundancy in coding: For example with 6 probe redundancy, the hybridization rate increase to 92% for a 3 position barcode. Of the barcodes that were completely hybridized, $72\pm 10\%$ ($n=50$, s.e.m.) were reconstructed correctly, consistent with the conventional fluorophore results and independent of mRNA species (Fig. 2 a,b, Supplementary Figs. 6-8).

For two-dimensional imaging the spatial barcoding scheme necessitates the linearization of mRNA. To do so, we compressed cells between coverglass slips, extending the mRNA through the lateral force of compression. Such treatment can be readily applied to single cell organisms and embryos, but may destroy spatially complex samples (Supplementary Note).

The principle drawback of spatial barcoding is its high-resolution requirement. It is difficult to use dyes that emit lower numbers of photons, such as Alexa 680 and Alexa 750, when high (~ 20 nm) intramolecular resolution is necessary. We anticipate that the continued development of improved fluorophores, 3D microscopy, and nucleic acid self-assembly²² will enable spatial coding to reach its full potential. In the meantime an alternative strategy which we call spectral barcoding is more appropriate for most applications.

Spectral Coding

In spectral barcoding (Fig. 2 e, f) the identity of mRNA is coded by the combination of fluorophores, ignoring spatial order. As long as all the colors are present (as determined by the integrated photon counts over the STORM imaging cycle) and can be identified above the cross-talk tolerances, the barcode assignment can be confidently made even if the total amount of collected photons is low. This comparatively low resolution requirement enables the use of dim fluorophores, as probes need only to be localized to within the ~100 nm area of a single mRNA (Table 1). Probes labeled with the same fluorophore can be distributed throughout an mRNA, making spectral coding more robust to heterogeneities in hybridization and partial degradation of mRNA. In contrast to spatial coding, molecules do not need to be linearized to be faithfully identified, potentially permitting other molecules, such as proteins, to be multiplexed under super-resolution imaging.

Profiling stress response genes in single yeast cells

As a proof of principle of our technique, we profiled transcripts from 32 known stress responsive genes (Supplementary Table 1) in single *S. cerevisiae* cells in response to extracellular Calcium stress. We have previously shown that the master transcription factor Crz1 translocates in and out of the nucleus in short (2-3 minute) well-defined pulses in the presence of extracellular Calcium²³. These pulses occur stochastically in time and activate target gene expression in a probabilistic fashion. In addition to Crz1, calcium stress also triggers the Msn2 pathway, a general stress response regulator that also pulses in its localization²³.

For mRNA profiling we selected 14 genes that are regulated by Crz1, 5 general stress response genes, as well as 13 other aging and stress markers²⁴. To ensure that we were observing the products of individual TF pulses, we fixed cells under conditions where the average interval between pulses²³ was longer than the typical mRNA lifetime.

To label these genes, we used spectral coding of combinations of 3 out of 7 photoswitchable dye pairs (${}_3C_7=35$). Barcode assignments are shown in Supplementary Table 1. Cells were imaged by STORM and transcript levels in individual cells quantified based on the abundances of the corresponding barcodes (Supplementary Fig. 9). The main concern with multiplexing a large probe set is the crosstalk among the barcodes. This can arise from several sources: fluorescent background from the cell, blinking of nonspecifically bound probes, crosstalk amongst the fluorophores, and errors in computational analysis. To rule out crosstalk and non-specific blinking, we only accepted fluorophore assignments at least 3 standard deviations from the measured crosstalk values (Supplementary Fig. 10). To rule out the possibility that random autofluorescence from cells would skew the measurements, we implemented an imaging scheme containing frequent frames without activation to reject high-frequency false-activation (Supplementary Fig. 11). In addition, we intentionally left certain barcodes unassigned and measured the rate of false positives from those barcodes to be 0.67 ± 0.84 (n=62 cells, s.d.) copies per cell, which represents the additive noise floor of our measurements.

To check the accuracy of our quantitation by super-resolution barcoding, we compared the mean expressions level with qPCR as well as single molecule FISH measurements, and obtained $R^2=0.95$ for both experiments (Fig. 3a, b). We found a 2 fold difference between mean copy number measured with smFISH vs super-resolution barcodes, which can be accounted for by the hybridization efficiency of our probe set and the high threshold levels used to reject false activations. For future probe set designs, a higher redundancy in probes will considerably increase the effective hybridization rate.

To determine if there is an appreciable bias introduced by a particular barcode scheme, we compared the expression levels measured by 2 different scrambled barcode schemes of 20 genes (Supplementary Table 2). The genes had a range of average copy numbers (from 1 to 10 copies per cell). Some genes (Cmk2, Npt1) are expressed at levels comparable or higher than the abundant tubulin subunits. The 12 remaining genes with low copy numbers (~ 1 per cell) were not measured since they are within the noise floor of our measurements. The two measurements agreed with an $R^2=0.88$ ($n=1871$ for the standard code, $n=1523$ for the switched code. Fig. 3c, Supplementary Table 2, Supplementary Figure 12) in 19 of the 20 compared genes. As switching scrambles the barcode assignments for genes with different expression levels, the consistent results in this experiment showed that barcodes of the high copy number genes did not leak into the low copy number barcodes and bias their readings.

Combinatorial regulation in the calcium response transcriptional network

Cells often need turn on the expression of a large battery of genes in response to external stress. We analyzed the expression of a battery of genes in the calcium stress regulon that respond to pulses of Crz1 and Msn2. In Fig. 4, each column represents a single yeast cell and the mRNA abundances measured for each gene under 50 mM Ca^{2+} stress. From this data it is clear that the transcriptional response to calcium varies widely among individual cells.

To characterize the heterogeneity in regulon expression in single yeast cells, we first examined the variability in the abundances of each mRNA species. The distribution of expression levels for most genes included a low basal state and a long-tailed high-expression mode corresponding to transcriptional bursts (Supplementary Fig. 13), similar to those observed in previous smFISH experiments in yeast²⁵. Since these transcriptional bursts are triggered by a TF pulse in our experiment, we asked whether our target genes burst synchronously with each other. Previous two-color smFISH experiments²⁶ in yeast found that targets of a constitutively active TF in the galactose response network as well as metabolic genes were well correlated ($R^2=0.5-0.7$). We found a range of correlation coefficients from -0.11 to 0.78 between pairs of calcium responsive genes (Supplementary Fig. 14,15), indicative of heterogeneous coordination throughout the regulon.

Given this heterogeneous regulon expression profile, we next asked whether there were subgroups of genes that tend to burst together and whether they correspond to different regulatory architectures. We resolved two distinct clusters for the Crz1 responsive genes ($P=0.09$, 0.08 respectively) (Fig. 4, Supplementary Fig. 16). In the first cluster, Crz1 genes were preferentially clustered based on their coexpression with Msn2 genes. The promoter sequences of many of these genes, such as *PUN1*, *YLR194C*, *RCN2*, *NPT1* contained Msn2

binding sites along with Crz1 binding sites. Genes in the other cluster predominantly contained only Crz1 binding sites, with the exception of *YPS1* and *PMC1*, which also contain Msn2 sites. The lower average correlations between pairs of Crz1 and combinatorially regulated genes ($R^2 = 0.36$, $SD = 0.16$, $n = 62$ cells) compared to those among pure Crz1 targets ($R^2 = 0.55$, $SD = 0.17$, $n=62$) or among the combinatorial targets ($R^2 = 0.52$, $SD = 0.11$, $n = 62$ cells) indicated different sources of inputs on the target genes.

Since Crz1 and Msn2 pulses are not synchronized in individual cells²³ at the low calcium (50 mM) levels used in this experiment, we hypothesized that the two clusters of cells in the data corresponds to cells responding to either a Crz1 pulse or a Msn2 pulse (Fig. 4, Supplementary Note). We tested this by knocking out the Crz1 and Msn2 pathway separately and visualizing the loss of the associated expression clusters (Supplementary Figs. 17-20). In cells treated with FK506, an inhibitor of the Crz1 phosphatase Calcineurin, the overall expression level was lower (Fig. 5a). Conversely, in the Msn2- Msn4- cells, all targets genes should be expressed in response only to Crz1 pulses (**Supplementary Note**). This is consistent with the observed coordination between pure Crz1 and combinatorial targets (Fig. 5e), as well as the disappearance of the cell cluster with only combinatorial genes expressed (Supplementary Fig. 18). This experiment indicates a modulatory effect for Msn2 on Crz1 target genes.

These experiments elucidated the combinatorial role of Msn2 and Crz1 on the stress response network and showed that single cell transcriptional profiling can be a valuable tool for identifying combinatorial control architecture in regulatory networks.

Discussion

Under a super-resolution microscope, cells can become virtual microarrays with giga-pixel information density. We have demonstrated two barcoding methodologies, spectral and spatial, and shown that by using 7 of the current super-resolution fluorophore pairs, transcripts from 32 genes can be detected simultaneously in single yeast cells using spectral barcoding.

Super-resolution barcoding can be dramatically scaled up with the techniques presented in this paper. We found that the typical transcript size is about 100 nm (Figs. 1 and 2,) and that transcripts are uniformly random distributed in yeast cells (Supplementary Fig.21) at a mean density of 1.9 ± 1.5 barcodes/ μm^2 ($n=2463$, s.d.). There are local regions that contain multiple mRNA barcodes within a diffraction-limited spot (Supplementary Fig.9, Supplementary Note) necessitating the use of super-resolution imaging. However, globally, super-resolution barcodes of the 32 profiled genes occupy less than 2% of the 2D super-resolution space available in a yeast cell, leaving a large amount of space open for further multiplexing. With one of the additional available emitters²⁷, ${}_{12}\text{C}_5=792$ genes can be spectrally coded.

Spectral Barcoding has a reconstruction fidelity dependent only on the hybridization of fluorophores, does not require linearization, has a low photon requirement and can be hybridized in a distributed pattern to increase robustness (Table 1). These advantages make it an excellent technique for barcoding mRNA and other molecules with unknown structures

with the existing SRM dye palette. In comparison, spatial barcoding has a localized hybridization pattern, a lower reconstruction fidelity due to the much higher photon requirements and the tertiary structure of the barcoded molecule (**Table 1**). It has the advantage of scaling and may be more applicable to chromosome and splice isoform barcoding.

Scaling up beyond 1,000 genes will require the implementation of 3D SRM²⁸ to improve the axial resolution by a factor of 20, and the synthesis of an additional far-IR fluorophore as an emitter (${}_{18}\text{C}_6=18,532$ genes can be spectrally coded with 1 additional emitter). At this higher barcode density, more sophisticated computational algorithms will also be necessary to identify barcodes in an intelligent and automatic fashion (Supplementary Note).

Super resolution barcoding provides several advantages over existing transcriptional profiling techniques. First, direct imaging of the sample preserves the spatial information both within cells and amongst cells. With the application of light sheet microscopy²⁹, this technique can be extended into optically thick samples without photobleaching associated with z-sectioning in epi-fluorescence microscopy. This advantage makes it a powerful tool in studying signaling in heterogeneous systems such as microbial ecosystems, tissue and embryos, where interactions among different cellular populations play an essential role in cellular decisions. Second, because of the single-molecule and *in situ* nature of the technique, the method is quantitative and avoids intrinsic bias in RNA extraction and conversion to cDNA. Lastly, many cells can be imaged simultaneously under a microscope quickly and throughput can be scaled up without considerable costs, compared to the high cost and long waiting time for sequencing single cells. After the initial cost of the probe set synthesis, the probe set can be hybridized many thousands of times to wild-type and mutant organisms. Super-resolution barcoding even at its current throughput can be a useful follow-up to existing high-throughput transcriptomics techniques by allowing genes of interest to be monitored with single cell resolution in spatially complex samples.

Lastly, the combinatorial labeling scheme can be applied to many types of molecules *in situ*. It is a short leap to consider combinatorial labeling of chromosomes and proteins³⁰, for single cell proteomics and ChIP experiments. We hypothesize that for many types of biochemical techniques, such as microarrays, there is an equivalent *in situ* single-cell experiment possible through super-resolution barcoding, removing the need for spatial separation traditionally performed by gels or dilution on a chip.

Methods

Probes design, Purification and Hybridization

25mer oligonucleotide probes (Biosearch Inc) were designed to match melting temperature whenever possible (Supplementary Table 3). FISH probes were designed with 2 base pair spacing between probes to allow efficient reactivation of the pair dyes, often leading to varying melting temperature between probes. Alexa Fluor 405, Alexa Fluor 488, and Cy3 were used as the activators and Cy5, Alexa Fluor 680, and Alexa Fluor 750 (Invitrogen) as the switchable dye. Labeling and purification of the probes followed the protocol in reference 16. Yeast cells were grown in YPD with 50 mM CaCl_2 and fixed in log growth

phase. Cells were also treated with 0.1% NaBH₄ for 30 minutes before the ethanol permeabilization step. We found the NaBH₄ treatment decreased the auto-fluorescent background of fixed yeast cells. Cells were stored at -20 °C in Eppendorf tubes and aliquoted out for hybridization experiments. Cells were hybridized with the probes overnight at room temperature in 20% formamide and 10% dextran sulfate. For all smFISH experiments, 12 probes (Supplementary Table 3) were hybridized to mRNA. For super resolution barcoding, sets of between 4 and 5, 6 to 7 probe pairs were used for Cy5, A680 and A750 respectively. After hybridization, cells were washed in 10% formamide and .2X SSC solution 3 times and imaged.

Imaging

For conventional epi-fluorescence microscopy, images were acquired on an Olympus IX81 with a 100x sapo objective with laser illumination at 532 nm, 594 nm, and 640 nm. Images were acquired with Micromanager software and an Andor Ikon-M DU934 BV CCD. Conventional fluorescence images were acquired in 3 different fluorescence channels (Semrock zero line filters). The centroids of the diffraction-limited FISH spots were calculated in each channel and the images were aligned by center of mass alignment of co-localized fluorescent spots between channels. This was sufficient for alignment without correcting for rotation and dilation.

Super-resolution imaging was performed on a Nikon TI-eclipse microscope with PFS autofocus lock. Standard super-resolution imaging buffers with glucose oxidase and β -mercaptoethanol were used¹³. The imaging lasers, a 640 nm laser along with a 30 mW 691 nm and a 30 mW 730 nm laser (Coherent Lasers) were brought to the sample through a 100x TIRFM objective. 405 nm, 473 and 556 lasers were used as activation lasers and imaging automation was controlled by Micromanager software. Two illumination pathways were used on the microscope, an imaging pathway containing the 640, 691 and 730 nm lasers and an activation pathway consisting of the remaining lasers. The activation pathway contained a automated filter wheel (Thorlabs) with ND filters .3, .6, 1 and 1.6 and an Uniblitz shutter, enabling automatic control of activation power by a custom written Micromanager script. No excitation filters were used in the microscope, enabling both activation and imaging wavelengths to reach the sample. Exposures times for all STORM movies were 150 ms for Cy5, 400 ms for Cy5.5 and 300 ms for Cy7.

For all STORM experiments, the imaging routine was designed to minimize crosstalk and photobleaching. To reduce activator crosstalk, every imaging routine sequentially used activation lasers from 556 nm, to 473 nm to 405 nm. As the absorption spectra of the activator dyes slightly overlap, this allowed us to minimize cross-activation by preferentially bleaching the overlapping dye before the routine reached the adjacent activation laser.

For the spatial coding images, samples were first imaged with only the 640 nm laser for 100 frames to switch off Cy5 and to determine the non-specific blinking rate. Then, 100 frames were acquired in each activation channel by co-illumination of the activation and the imaging lasers. This reduced the crosstalk among the different activation channels.

For the spectral coding images, the samples were first bleached in all imaging channels for 6 frames. Samples were imaged in order of the activators, starting at 556 nm to 473 nm and to 405 nm. For the 556 nm laser, the microscope first imaged the 730 nm followed by either the 640 nm or 691 nm laser for 48 cycles. This pattern minimized the Cy5 and A680 signal loss due to bleaching of both dyes by the 640 nm and 691 nm lasers. For the remaining activation lasers, the microscope cyclically imaged the 730 nm laser followed by the 640 nm laser. Samples are only illuminated with the activation light in the A750 channel. This imaging pattern minimized photobleaching of A750 while still allowing us to observe adequate activation of the A680 and Cy5 probe pairs. Throughout the imaging routine, for every 4 cycles of the imaging lasers, 2 cycles without the activator lasers were acquired. This enabled us to rule out much of the false positive and nonspecific blinking events in the images. Activation powers were selected to maximize activation rate while avoiding crosstalk amongst the activators. Following the imaging cycle with all emitters, the 640 nm laser was continuously used to image Cy5 for another 30 frames of specific activation with an ND filter added to the pathway to bring the activation laser power to 50% of the original activation. The acquisition sequence is illustrated in Supplementary Fig. 11.

The activation lasers were controlled by an Arduino microcontroller board and a servo motor shutter. Fluorescent beads (Invitrogen F-8810) were used as fiducial markers to correct for stage drifts. The microscope stages (Prior and ASI) were automated and controlled by acquisition software to enable multi-position imaging.

Analysis

Images from diffraction limited and super-resolution experiments were analyzed with a custom written Mathematica script, available upon request. In the analysis, the beads were first aligned to determine the stage drifts. Beads emit on the order of 50,000 photons per image, and could be localized to a few nanometers. Beads close to the cells were eliminated from analysis as the switching of fluorophores in cells can disrupt the bead alignment. Then, fluorophores were selected from each image by intensity thresholding (600 photons for cy5, 400 photons for A680 and 250 photons for A750), and their centroid calculated by Gaussian fitting. We did not reject activation events that involved multiple fluorophores in the analysis since they came predominantly from single clusters and allowed us to preserve more of the photons collected for the analysis. In principle, multiple activation frames can be compared to neighboring frames to extract localization information.

To determine which of the barcode colors are present instead of nonspecific activation, we compared the number of activation events occurring in the specific activation channel versus the activations observed in frames with no activation. If the frequency of nonspecific activation events exceeded that of specific activation events, then that channel was rejected. As several of the activators and emitter channels can crosstalk into other channels, we quantified the crosstalk ratio and rejected activation events if they fell below our thresholds (further discussion in the Supplementary Note). Switching events that spatially cluster together were grouped to display the resolved barcode. For spectral barcodes, activations were clustered together on a 184 nm grid for simplicity since most RNAs are contained within a diameter of 100 nm. Activation events near grid vertices were assigned to a

neighboring region containing localizations of the same fluorophore pair. For 3 color spatial barcodes, the center color was determined by finding the position that was not one of the two localized colors separated by the longest distance. Cell positions were determined by manual segmentation. Barcodes were collected and tabulated for each single cell. Cross-correlation was calculated using the correlation function in Mathematica and the standard errors were calculated from re-sampling the data 100 times.

QPCR Measurements

For the RT-PCR experiments, *S. cerevisiae* were grown in YPD media and were induced with 50mM CaCl₂ for two hours. The cells were then spheroplasted with zymolyase, RNase inhibitor and BME. The RNA isolated using the Trizol Plus RNA Purification System (Invitrogen). The extract was treated with recombinant Dnase I (Roche) to digest genomic DNA. Reverse transcription of the RNA was performed using the iScript Reverse Transcription Supermix (Bio-Rad). Aliquots of the resulting cDNA were diluted 1:10 and 1:100 with water. Quantitative PCR was performed using Maxima Sybr Green and Fluorescein qPCR Master Mix (Fermentas) on a Bio-Rad CFX machine. Quantitative PCR was performed on each gene with the undiluted cDNA, 1:10 dilution cDNA and 1:100 dilution cDNA, with each dilution repeated in triplicate (Figure 3). The PCR efficiency for each gene was calculated from a linear fit of the three dilution points. The abundance of the RNA was determined from the amplification efficiency. Primer sequences are presented in Supplementary Table 4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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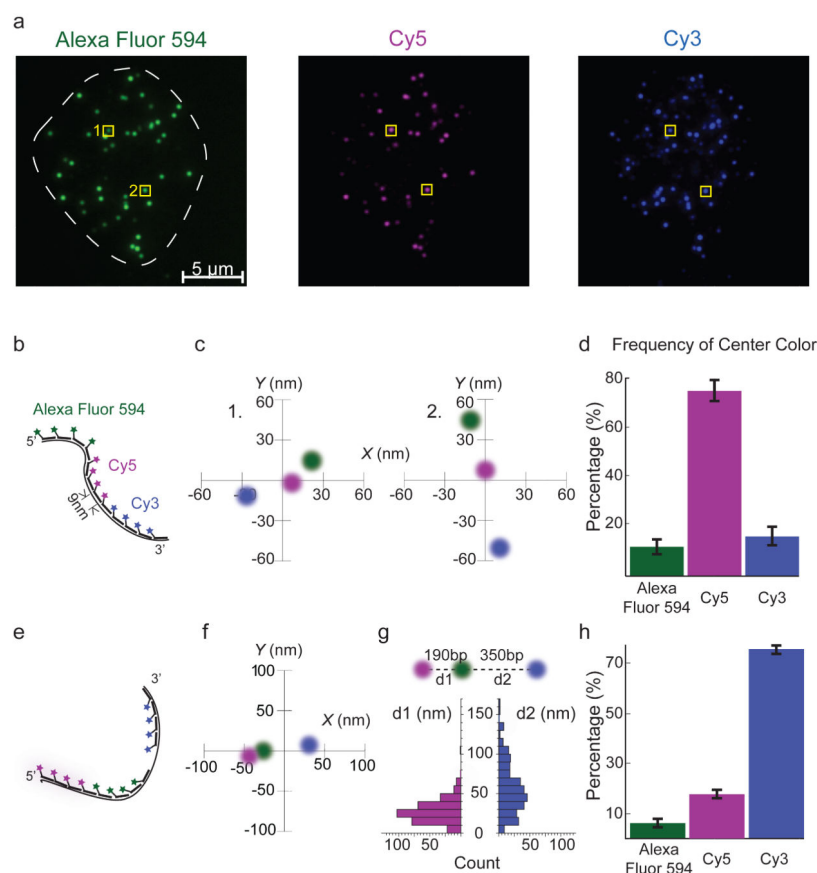
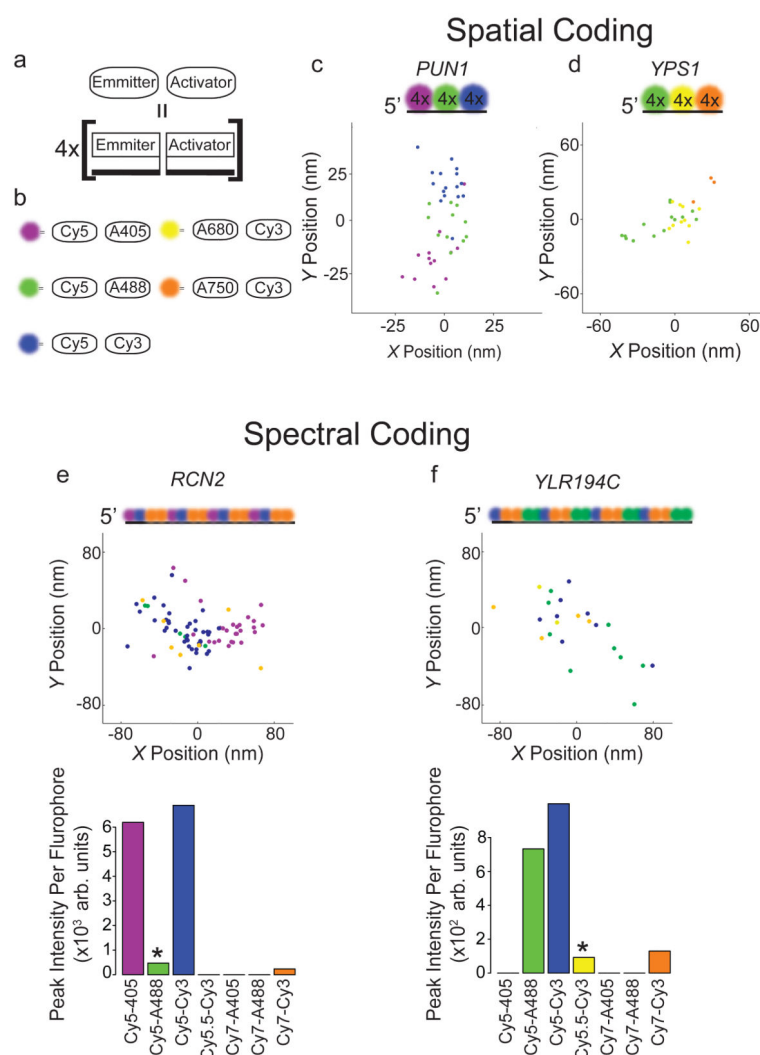


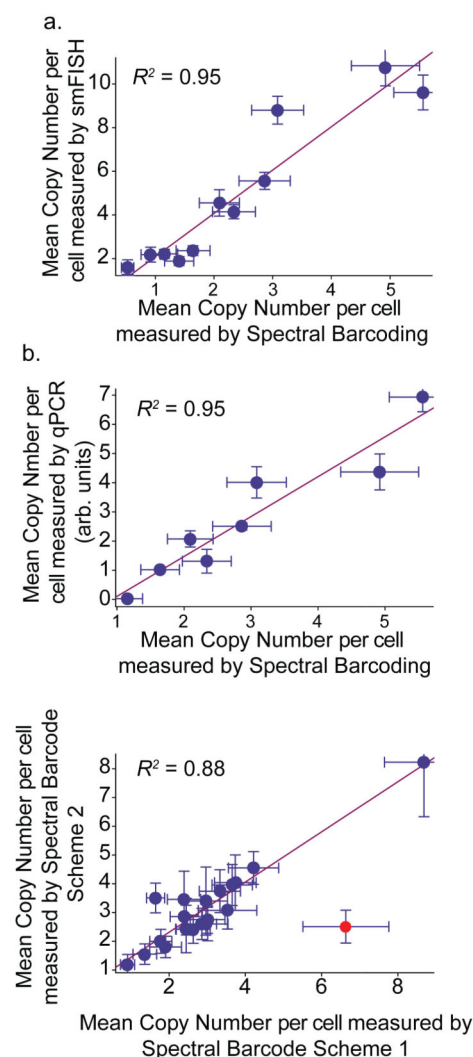
Figure 1.

Spatial ordering of fluorophores on mRNAs can be resolved by gaussian centroid localization. (a). Fluorescence images of *PUN1* probes hybridized in a single budding yeast cell, shown in each channel. (b). Schematic of labeled 25mer oligonucleotides hybridized to *PUN1* mRNA. (c). Reconstructions of the centroids of spots 1 and 2 following localization by Gaussian fitting and image alignment. (d). The percentage of co-localized *PUN1* three-color dots that can be reconstructed in the above image (a) with the correct barcode ($n=28$, Correct Order= $74\pm 8\%$, s.e.m.). (e). Schematic of probe-set hybridized to GFP mRNA with different order and distances between the probes positions. (f). Gaussian fitting reconstruction of this probe set. (g). The distance between the resolved centroids positions ($d1=27.93\pm 14$ nm, $d2=56\pm 33$ nm s.d.) is proportional to the intramolecular distance between barcode positions (190 and 350bp). (h). The frequency of barcode identification for this probe set ($n=327$, correct order= $76\pm 2\%$, s.e.m.).

**Figure 2.**

Super-resolution imaging enables combinatorial labeling of individual transcripts. **(a).** Schematic of super-resolution barcoding scheme. For each super-resolution probe pair, four acceptor-emitter pairs are hybridized in sequence for redundancy. **(b).** Each barcode color consists of an activator (Alexa Fluor 405, Alexa Fluor 488, and Cy3) labeled oligo adjacent to an emitter (Cy5, Alexa Fluor 680 and Alexa Fluor 750) labeled oligo. **(c).** *PUN1* mRNA 3 position spatial barcode. The order of the probes is shown schematically in the cartoon. A localization scatterplot in which each dot represents an activation of a fluorophore pair is shown. **(d).** *YPS1* mRNA 3 position spatial barcode with 3 different emitters. **(e).** *RCN2* mRNA spectral 3 Position Barcode. Probe Positions are scattered throughout the mRNA, enabling robust hybridization. Bar height in histogram is equal to the number of integrated peak pixel counts detected for each fluorophore pair over time. Cy5-A405, Cy5-A488, Cy5-Cy3 and A750-Cy3 are detected with 6195, 471, 6881 and 235 integrated counts respectively. Cy5-A488 (green and asterisk) is present due to cross-talk from Cy5-Cy3 (blue), and rejected based on the threshold measurements in Supplementary Figs. 10. Note that the A750 based dye pairs give fewer photons than Cy5 dye pairs, but are readily

detected with less crosstalk. **(f)**. YLR194c mRNA spectral 3 position barcode. Cy5-A488, Cy5-Cy3, A750-Cy3 and A680-Cy3 are detected with 773, 999, 130 and 92 integrated peak counts. A680-Cy3 (yellow and asterisk) was rejected due to crosstalk from Cy5-Cy3.

**Figure 3.**

Validation of mRNA quantitation by super-resolution barcoding. **(a)**. Comparison of smFISH results with super-resolution barcodes gives an $R^2 = 0.95$, with a slope of 2.05. 11 genes were FISHed, including 8 *crz1* specific genes, 1 *Msn2* target gene, and 2 aging and stress genes. **(b)**. For the qPCR experiment, 8 *Crz1* genes were quantified. **(c)**. Robustness of mRNA quantitation measured by two different barcode schemes. Mean copy-number measurements for barcoding schemes are displayed as points in the scatterplot. A regression with an R^2 value of 0.88 was obtained following removal of the one outlier connoted by a red circle. The outlier was removed due to its high cook's distance of 2.1 (Supplementary Fig. 12).

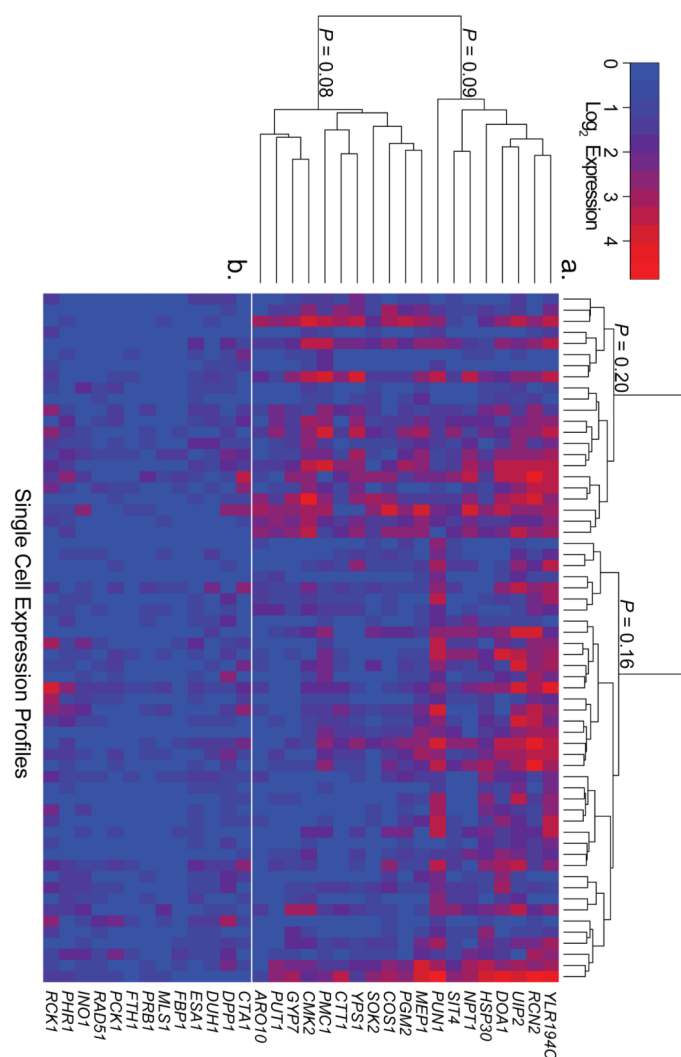


Figure 4. Single cell expression profiles of 32 mRNAs. Each column corresponds to the expression profile of a single yeast cell. Cells, and genes in **(a)** are clustered using agglomerative hierarchical clustering on the correlation between species using Ward's criterion. The P -values for the secondary clusters are given by bootstrap resampling and placed adjacent to these clusters. **(a)**. Genes responsive to Crz1 and Msn2. Genes can be broadly clustered into two classes, one largely containing genes regulated by both Crz1 and Msn2 ($P=0.09$, upper cluster) and one largely containing genes regulated by Crz1 ($P=0.08$, lower cluster). Cells are grouped in two distinct clusters, one showing correlations amongst the expression of all genes regulated by Crz1 ($P=0.2$, left cluster), the other with large expression correlations amongst combinatorial genes ($P=0.16$, right clusters). **(b)**. Additional measured genes are shown.

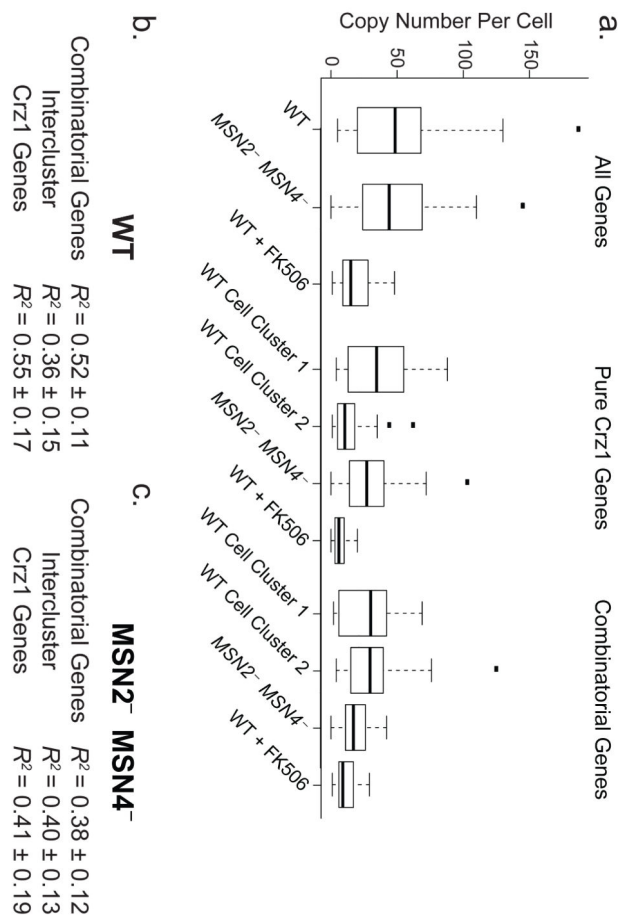


Figure 5. Msn2 and Crz1 combinatorially affect target regulons. All data in this analysis comes from the genes in Fig. 4a, genes displayed in Fig. 4b are not included in these analyses. **(a).** Total number of regulon-specific mRNAs in single yeast cells shown in box plots. Pure Crz1 targets are expressed in WT and in the Msn2⁻ and Msn4⁻ cells, but are repressed in FK506 treated cells. WT cells are clustered into two groups (Fig. 4a), WT Cell Cluster 1 and WT Cell Cluster 2, corresponding to cells with Crz1 or Msn2 pulses respectively. Combinatorial targets are repressed in the Msn2⁻ and Msn4⁻ as well as in the FK506 treated cells. **(b-c).** Averaged correlation coefficients between pairs of genes in the combinatorial or pure Crz1 target clusters. The detailed pairwise gene expression scatter plots are shown in Supplementary Figs. 14,15 .

Table 1

Comparative advantages of SRM barcoding techniques.

Barcode Type	Hybridization Pattern	Spatial Reconstruction Fidelity	Resolution Requirement	Minimum required Fluorophore Emission	Linearization required	Multiplex Scaling
Spectral	distributed	100%	100 nm	~400 photons	No	$p!/(p-n)!$
Spatial	localized	74%	20 nm	~3000 photons	Yes	$p!/(p-n)!/2$

*Calculations of multiplex scaling use p as the number of fluorophores and n as the number of positions.

Single-cell systems biology by super-resolution imaging and combinatorial labeling

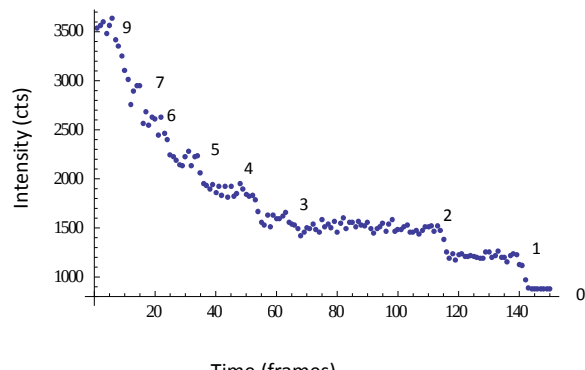
Eric Lubeck & Long Cai

Supplementary File	Title
Supplementary Figure 1	Photobleaching and reconstruction with conventional fluorophores
Supplementary Figure 2	Gaussian fitting reconstructions of <i>MDN1-GFP</i> (14kb) mRNA tagged with the barcode Cy5-A594-Cy3
Supplementary Figure 3	Gaussian fitting reconstructions of <i>MDN1-GFP</i> (14kb) mRNA tagged with the barcode A594-Cy5-Cy3
Supplementary Figure 4	Gaussian fitting reconstructions of <i>PUN1</i> (14kb) mRNA tagged with the barcode A594-Cy5-Cy3
Supplementary Figure 5	Median Photons per fluorophore
Supplementary Figure 6	STORM reconstructions of <i>PUN1</i> (14kb) mRNA
Supplementary Figure 7	Single cell STORM reconstructions of <i>PMCI</i> 3 position barcodes Cy5/A405-Cy5/Cy3-Cy5/A488 (Blue-Red-Green)
Supplementary Figure 8	Single cell STORM reconstructions of <i>YPS1</i> 3 position barcode with Cy3 paired with Cy5 (blue), A680 (purple) and A750 (pink)
Supplementary Figure 9	Reconstructions of 32 Spectrally barcoded mRNAs in single cells
Supplementary Figure 10	Barcode crosstalk measurements
Supplementary Figure 11	Imaging Scheme
Supplementary Figure 12	Statistical analysis of barcode scramble experiment
Supplementary Figure 13	Distribution of expression levels for each Crz1 and Msn2 gene
Supplementary Figure 14	WT pairwise gene correlations
Supplementary Figure 15	Numeric reference for shaded WT correlations shown in Supplementary Figure 10
Supplementary Figure 16	WT cell clustering with the aging genes included
Supplementary Figure 17	Heat maps of single cell gene expression levels under FK506 treatment
Supplementary Figure 18	Heat maps of single cell gene expression levels in Msn2- Msn4-deleted cells
Supplementary Figure 19	Msn2- Msn4- pairwise gene correlations
Supplementary Figure 20	FK506 pairwise gene correlations
Supplementary Figure 21	Barcodes density in the current 32gene multiplex
Supplementary Table 1	Barcode Assignments for the 32 genes profiled
Supplementary Table 2	Circularly permuted barcodes used in switched barcode experiment (Figure 3 and S5)
Supplementary Table 3	Oligo probes for smFISH
Supplementary Table 4	qPCR Primers
Supplementary Note	

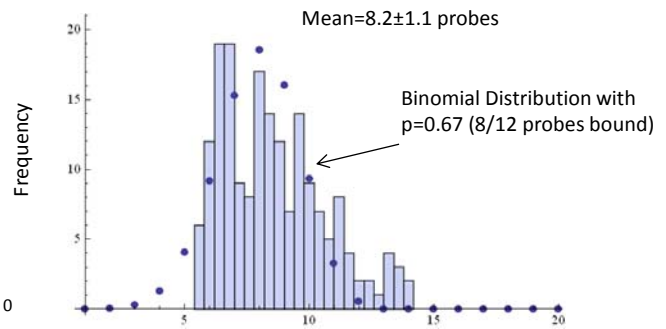
Single cell systems biology by super-resolution imaging and combinatorial labeling

Eric Lubeck and Long Cai

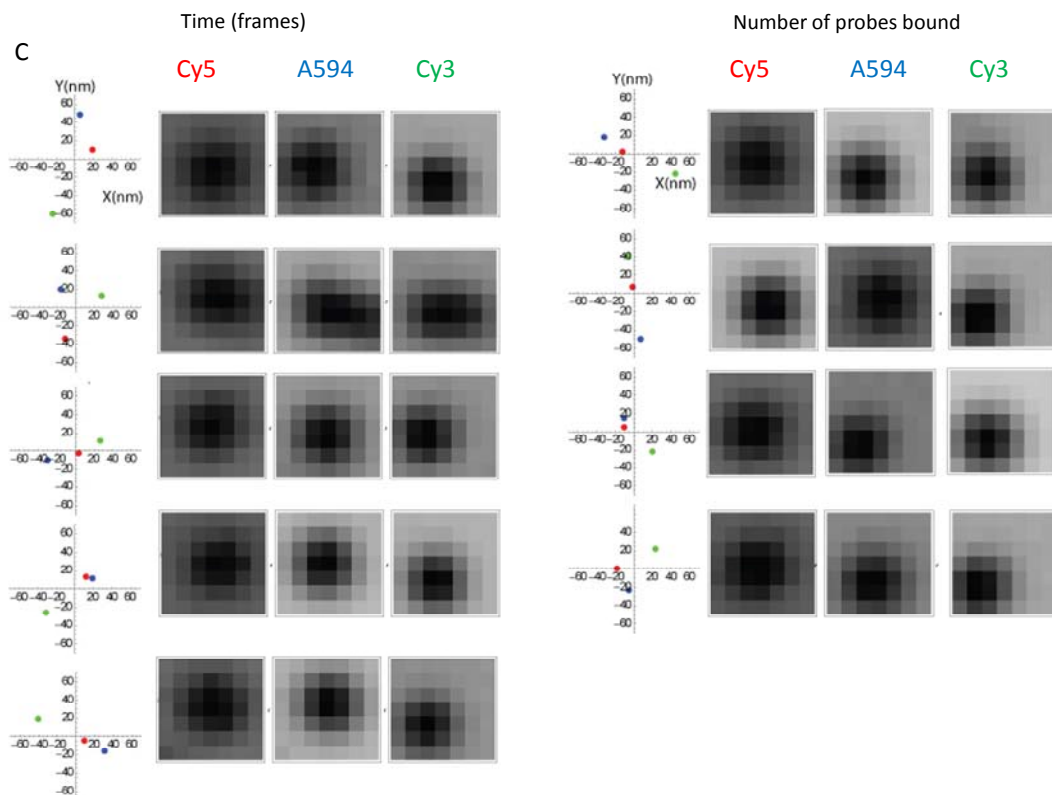
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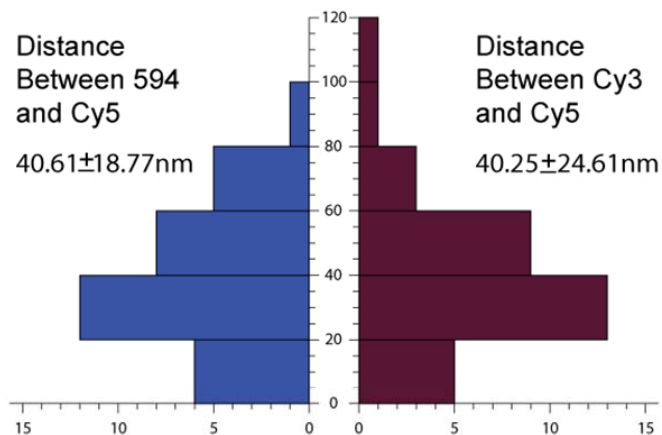
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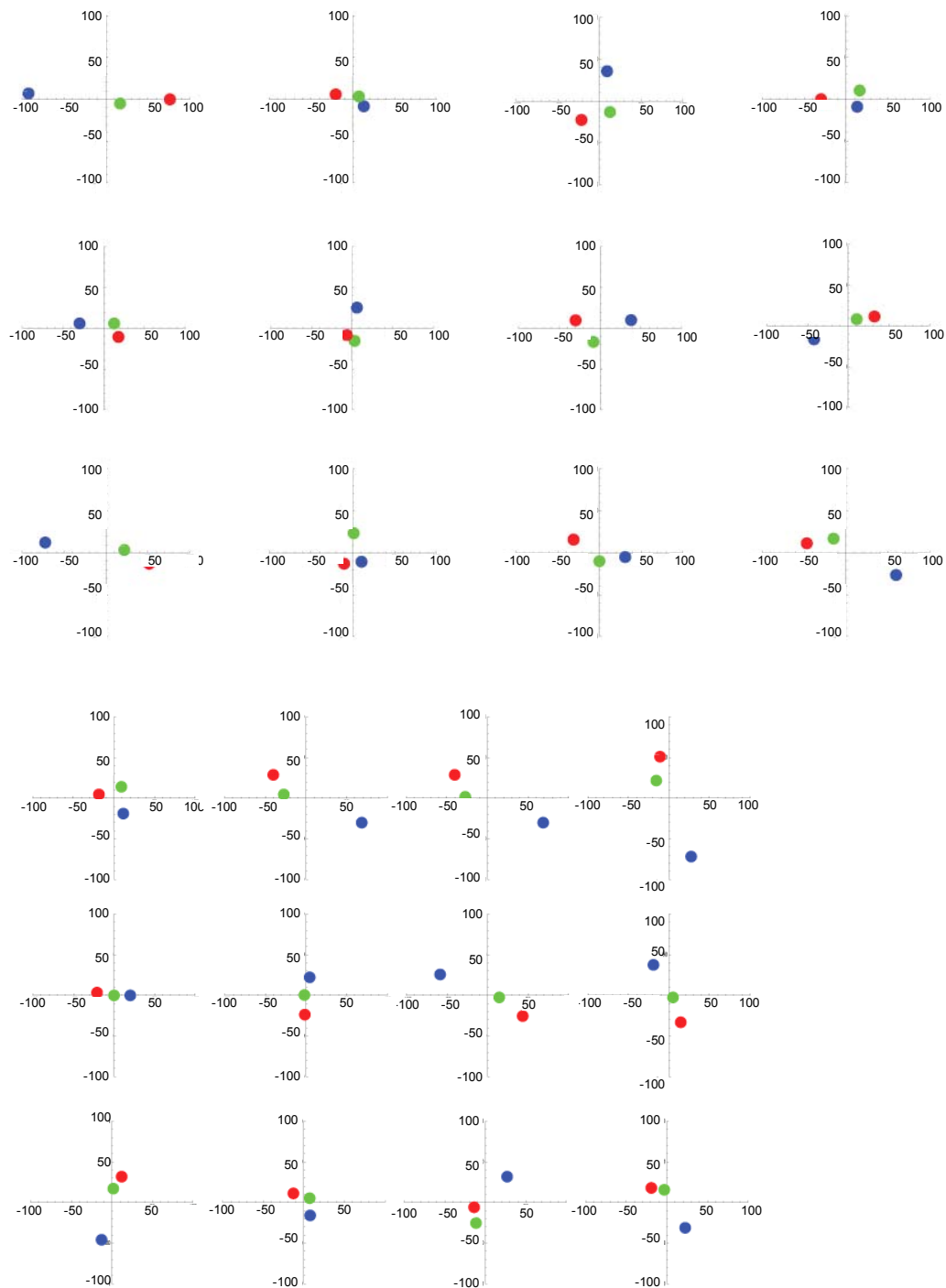
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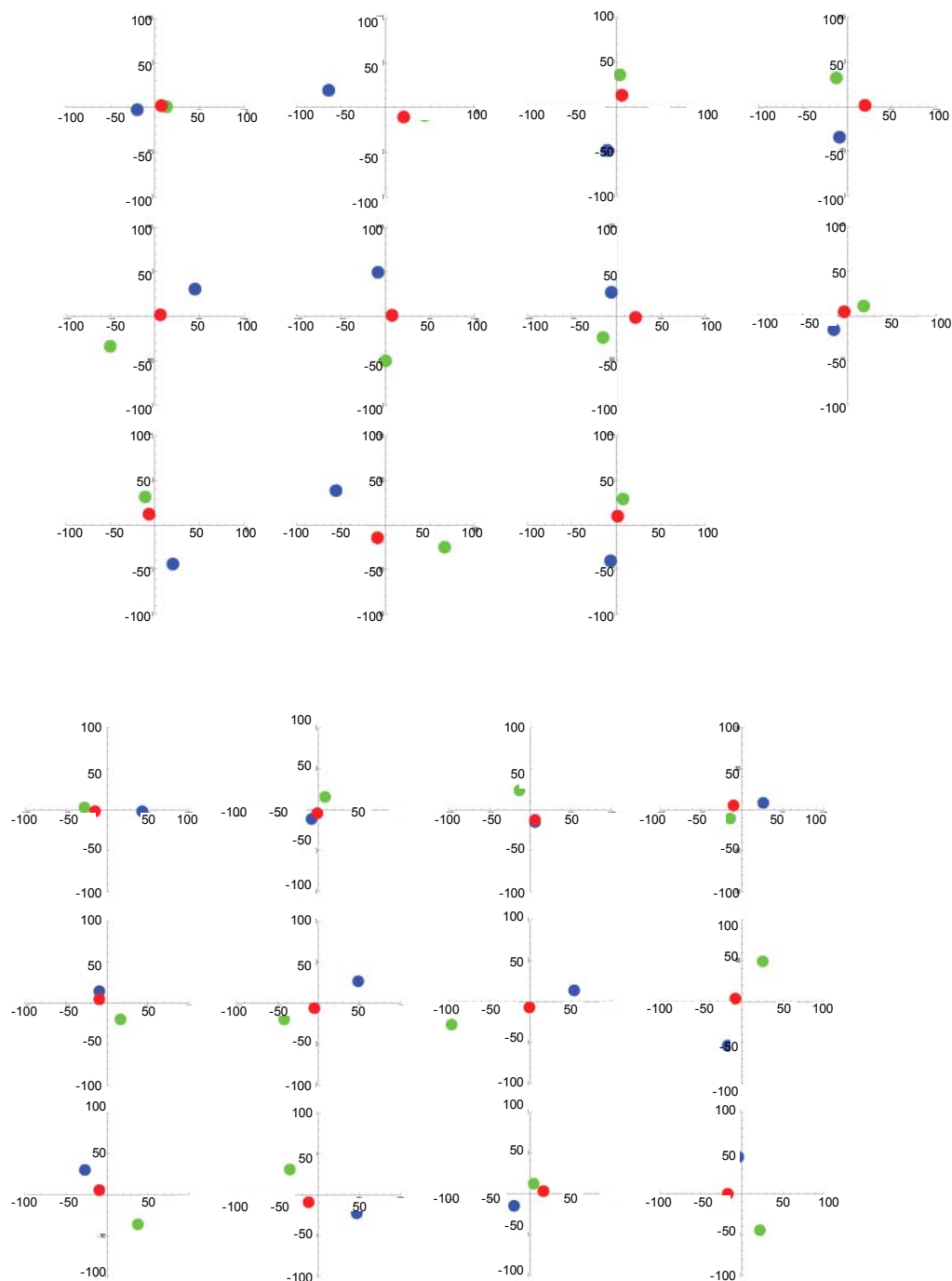
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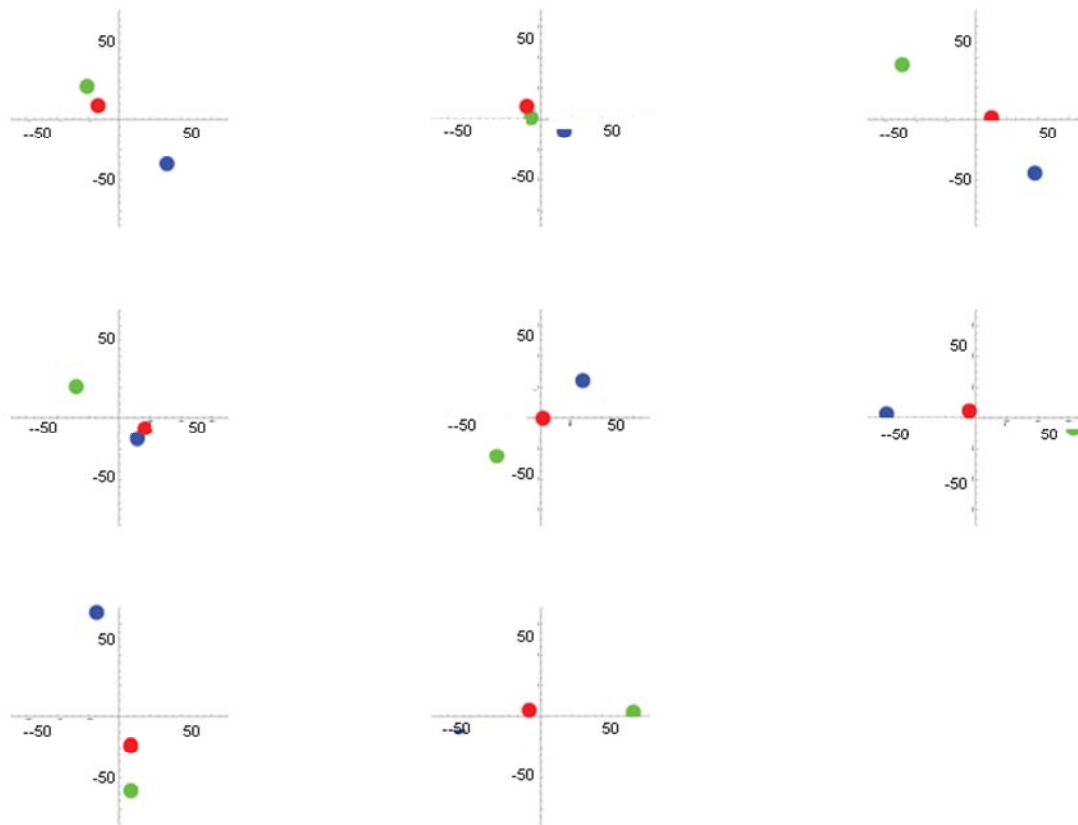
Supplementary Figure 1. Photobleaching and reconstruction with conventional fluorophores. **(A)** Sample photobleaching traces. *CMK2* mRNA was hybridized with twelve 27 mer probes labeled with Cy3. The sample was illuminated with a 532nm laser for 150 frames. No antibleaching buffer was used. Stepwise drops in fluorescence intensity correspond to photobleaching of single fluorophores. The intensities of fluorophores was not uniform, possibly due to micro-environment and homo-FRET quenching. On average each step corresponds to ~300 cts, with a background of ~900 cts. The initial intensities suggest, in both traces, 8-9 probes out of the 12 probes were bound to the mRNA, corresponding to ~2/3 hybridization efficiency for each probe. **(B)** Distribution of hybridization efficiencies for the *CMK2* probe set. The number of probes bound is determined from the initial intensities of dots observed prior to photobleaching divided by the average step size. The mean number of probes bound was 8.2 ± 1.1 probes. This distribution is overlayed with a binomial distribution with the probability of each probe bound at 67%, corresponding to 8 out 12 probes bound on average. **(C)** FIONA reconstructions of barcodes on *PUN1* mRNAs in a single cell in Fig1. 5' modified *PUN1* probes were used. The intensity profiles of the dots in each channel are shown in the right panels, corresponding to Cy5, A594, and Cy3 channels. The reconstructions from Gaussian fitting of the intensity profiles are shown in the left. mRNAs with intensities above thresholds in all three channels are selected in order to increase localization accuracy. **(D)** Spatial separation between terminal and center positions of the barcode. The distances between the A594-Cy5 and Cy3-Cy5 probe positions was both ~240bps, reflected in the symmetrical mean physical distances observed.



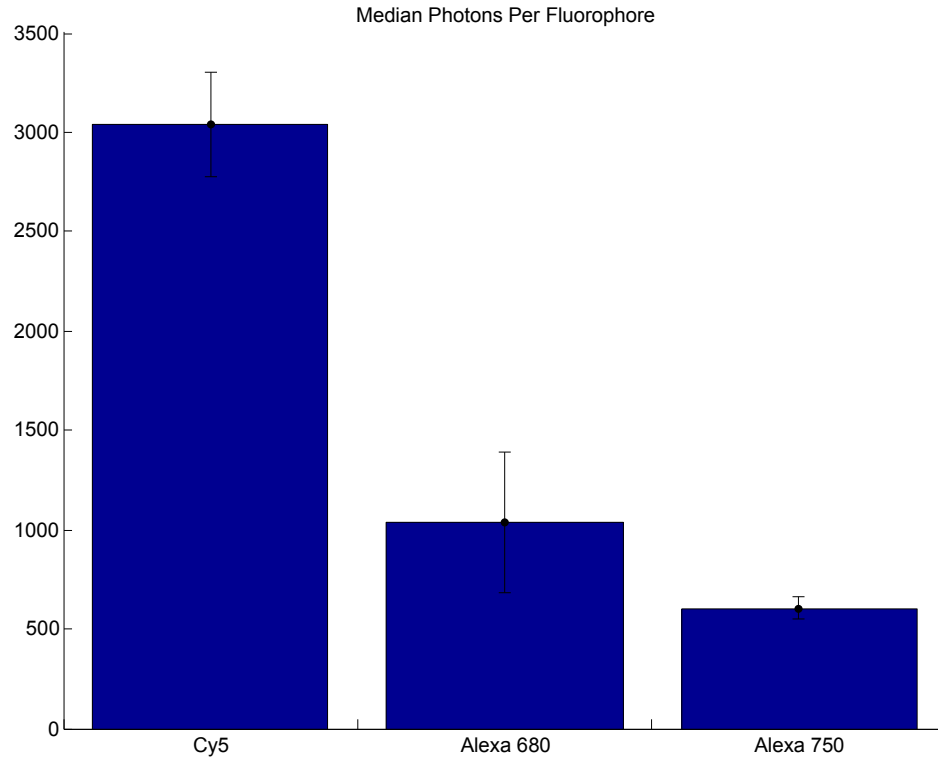
Supplementary Figure 2. Gaussian fitting reconstructions of MDN1-GFP (14kb) mRNA tagged with the barcode Cy5-A594-Cy3. Barcode represented as Red-Green-Blue. Axes are in nm. Reconstructions are taken from all of the dots from one image. The reconstruction rate is $92 \pm 4.4\%$.



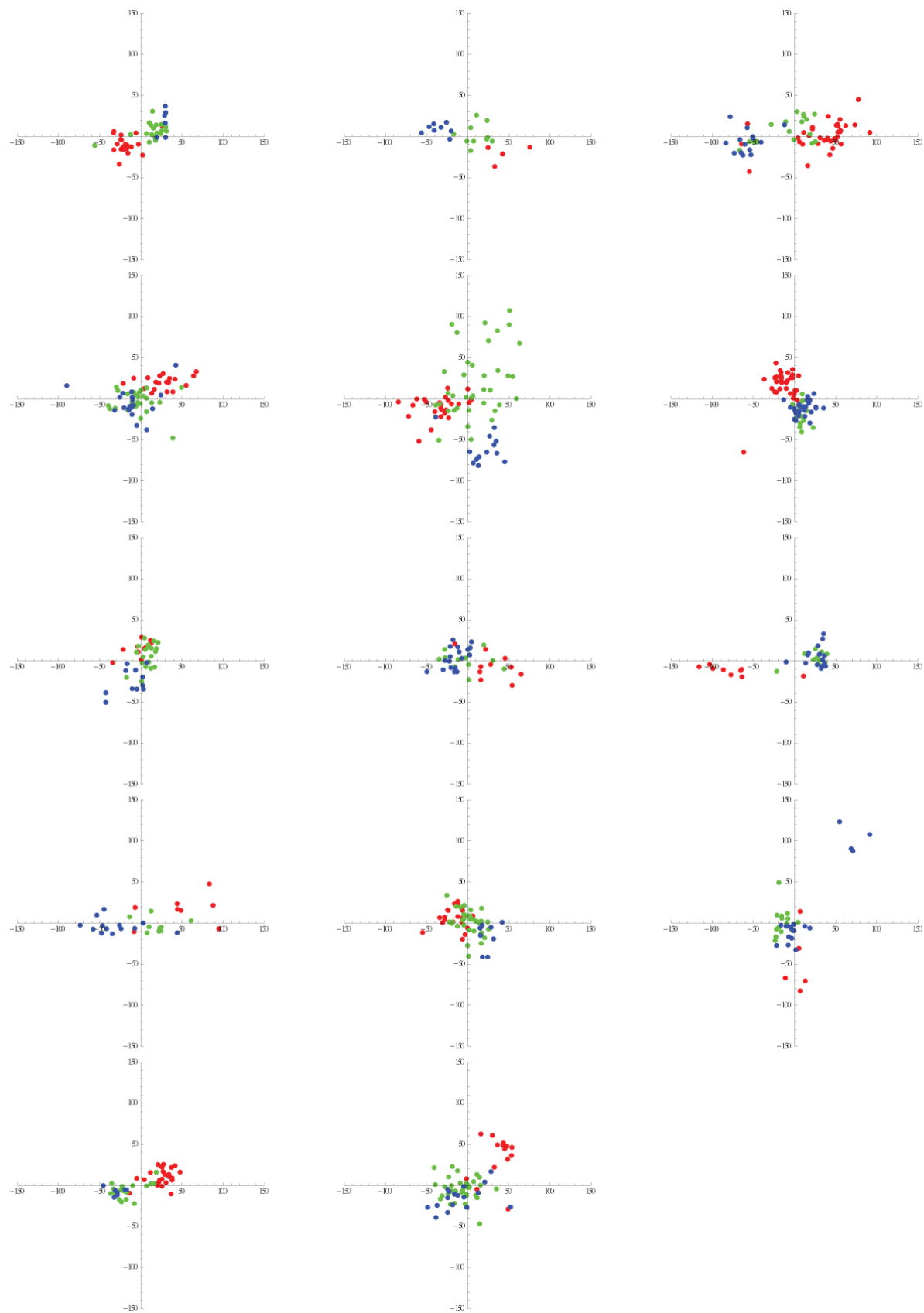
Supplementary Figure 3. Gaussian fitting reconstructions of *MDN1-GFP* (14kb) mRNA tagged with the barcode A594-Cy5-Cy3. Barcode represented as Green-Red-Blue. Order switched from **Supplementary Figure 2**. Axes are in nm. Reconstructions are taken from all of the dots from one image. The reconstruction rate is $72 \pm 7.5\%$ of the time.



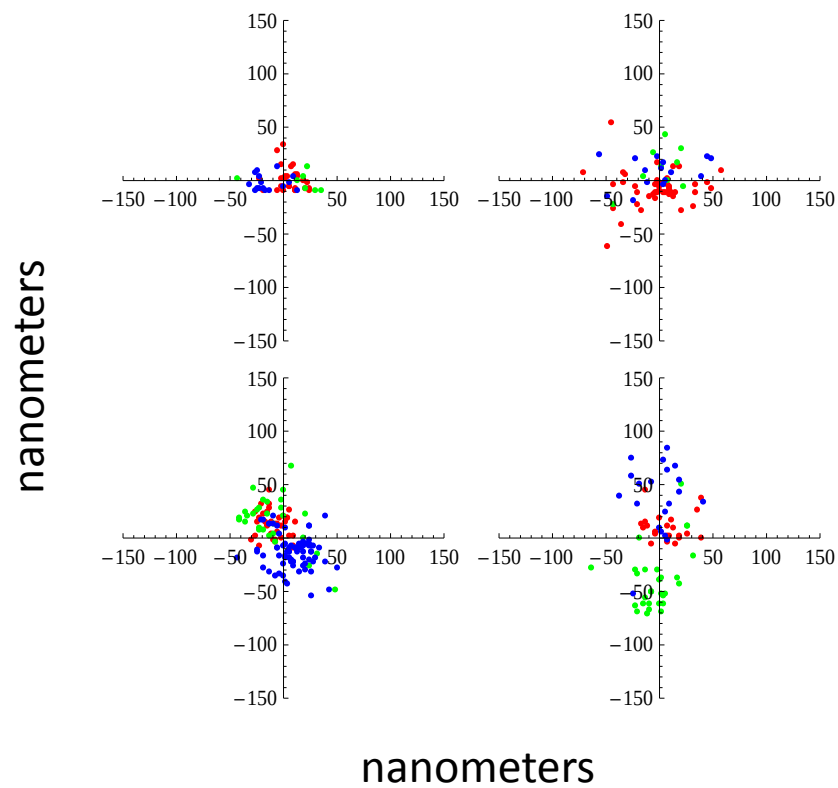
Supplementary Figure 4. Gaussian fitting reconstructions of PUN1 (14kb) mRNA tagged with the barcode A594-Cy5-Cy3. Barcode represented as Green-Red-Blue Axes are in nm.



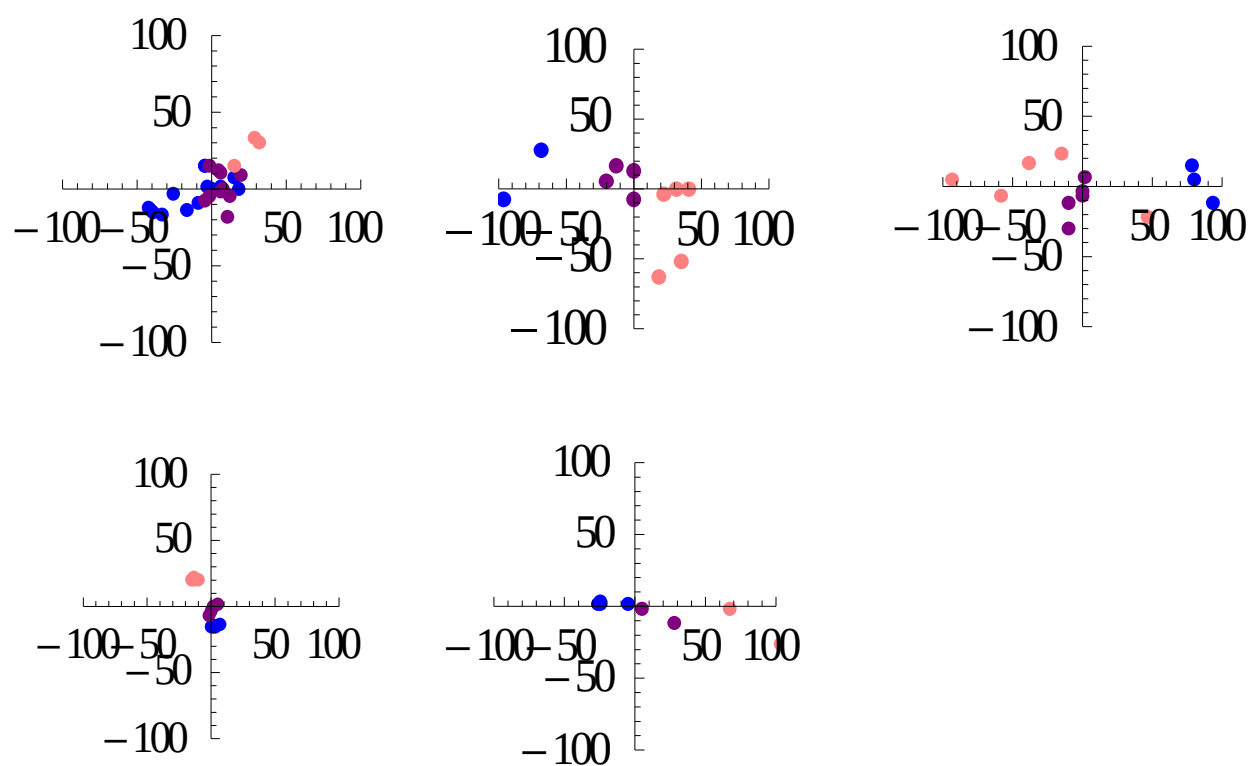
Supplementary Figure 5. Median Photons per fluorophore. Error bars determined by the 1000 replicates of the bootstrap standard deviation. In principle, Cy5, A680 and A750 should give a localization accuracy of 6.2, 11.3 and 15.1 nm. $n = 197, 71$ and 250 for Cy5, A680 and A750. In practice the localization accuracy within a barcode cluster was significantly lower than this due to the additive error of aligning multiple fluorescent beads ($SD \sim 5\text{-}15$ nm). Localization accuracies within a single color position (4 probes) in a spatially labeled barcode as measured by SD were 23.5 ± 18.4 nm, 21.71 ± 15.0 nm and 21.5 ± 16 nm for cy5, A680 and A750. $n=838, 552$ and 103 barcodes respectively.



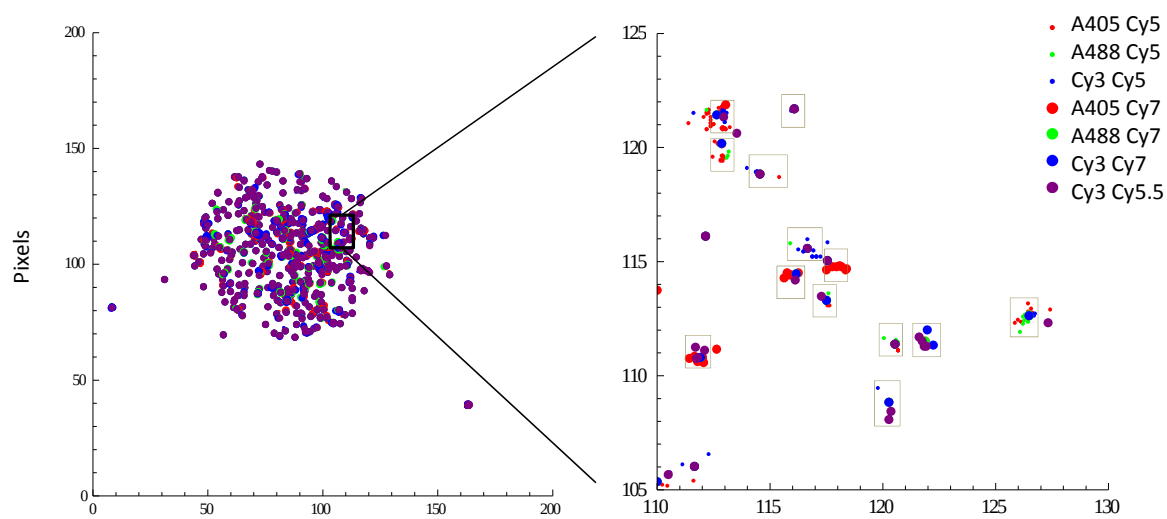
Supplementary Figure 6. STORM reconstructions of *PUN1* (14kb) mRNA. B. Each barcode color consists of an activator (Alexa 405, 488, and Cy3) labeled oligo adjacent to a 5' emitter (Cy5, A680 and A750) labeled oligo. Blue is A405 and Cy3, green is A488 and A680, red is Cy3 and A750. Green should be the center color. The axis for each figure goes from -150 nm to 150 nm in the X and Y dimensions.



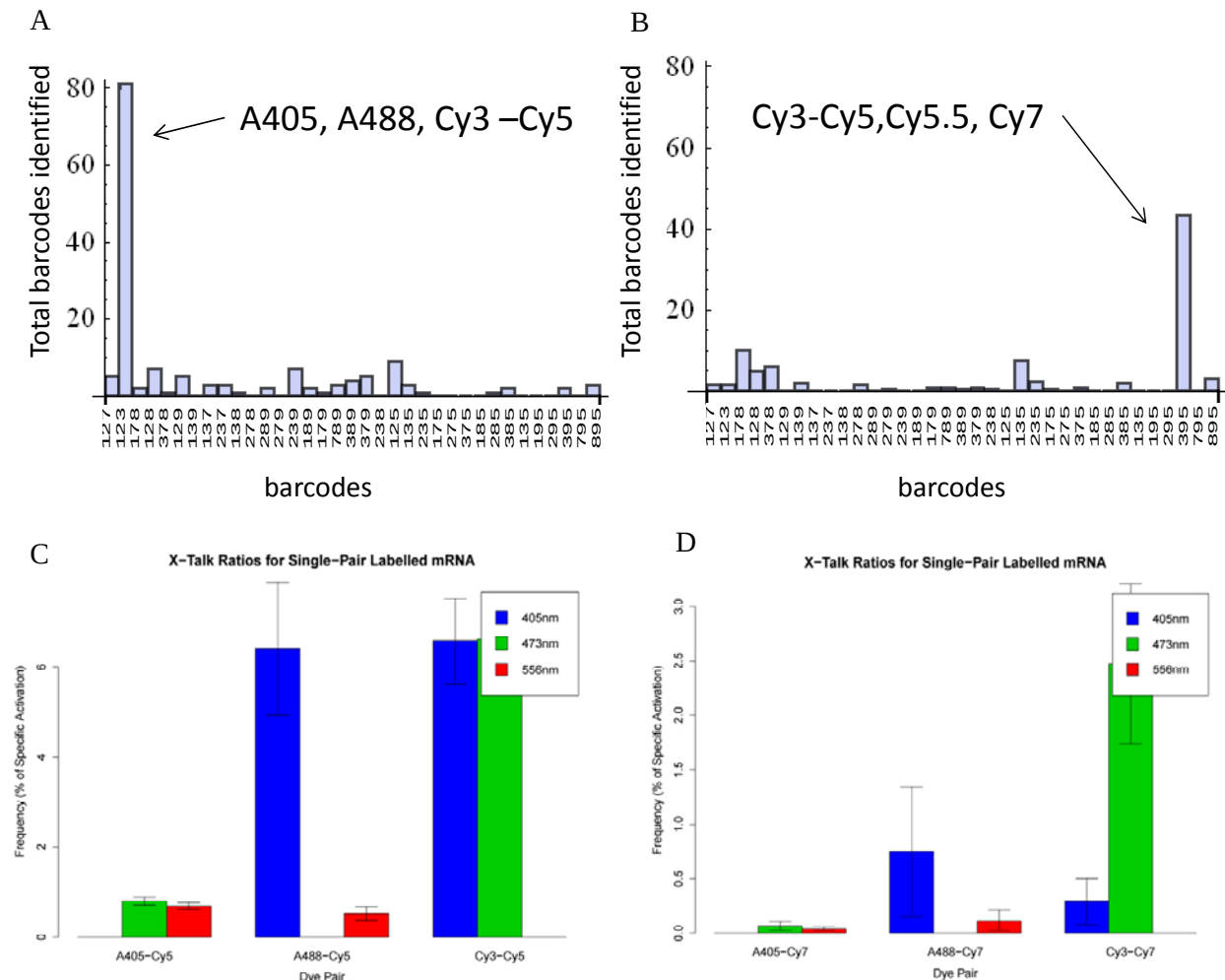
Supplementary Figure 7. Single cell STORM reconstructions of *PMC1* 3 position barcodes Cy5/A405-Cy5/Cy3-Cy5/A488 (Blue-Red-Green). Axes are in nm.



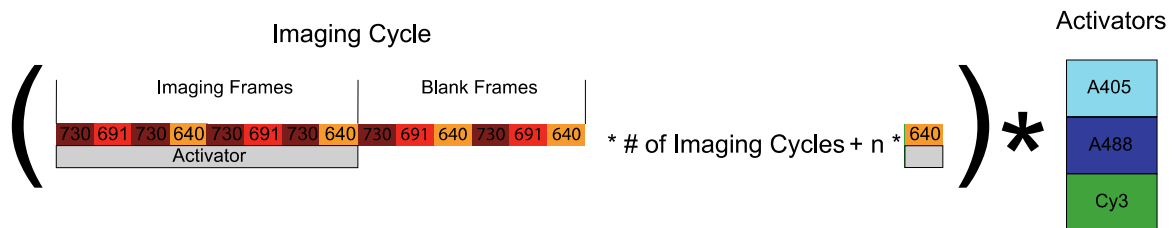
Supplementary Figure 8 . Single cell STORM reconstructions of *YPS1* 3 position barcode with Cy3 paired with Cy5 (blue), A680 (purple) and A750 (pink). Barcode order should read Cy3/Cy5-Cy3/A680-Cy3/A750. Axes are in nm.



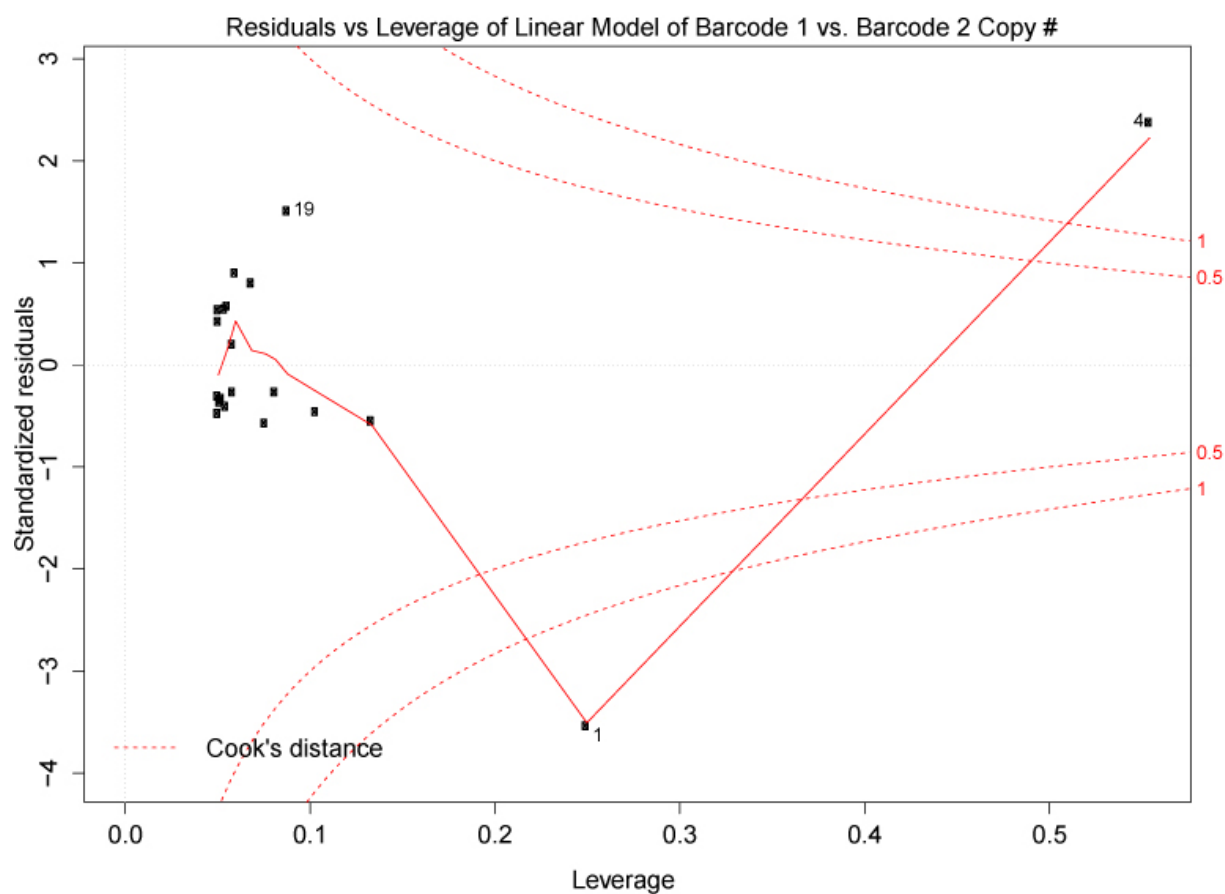
Supplementary Figure 9. Reconstructions of 32 spectrally barcoded mRNAs in single cells. Axes are in pixels, each corresponding to 130nm. The right panel shows a zoomed plot of a region in the cell. Individual mRNAs are shown in boxes.



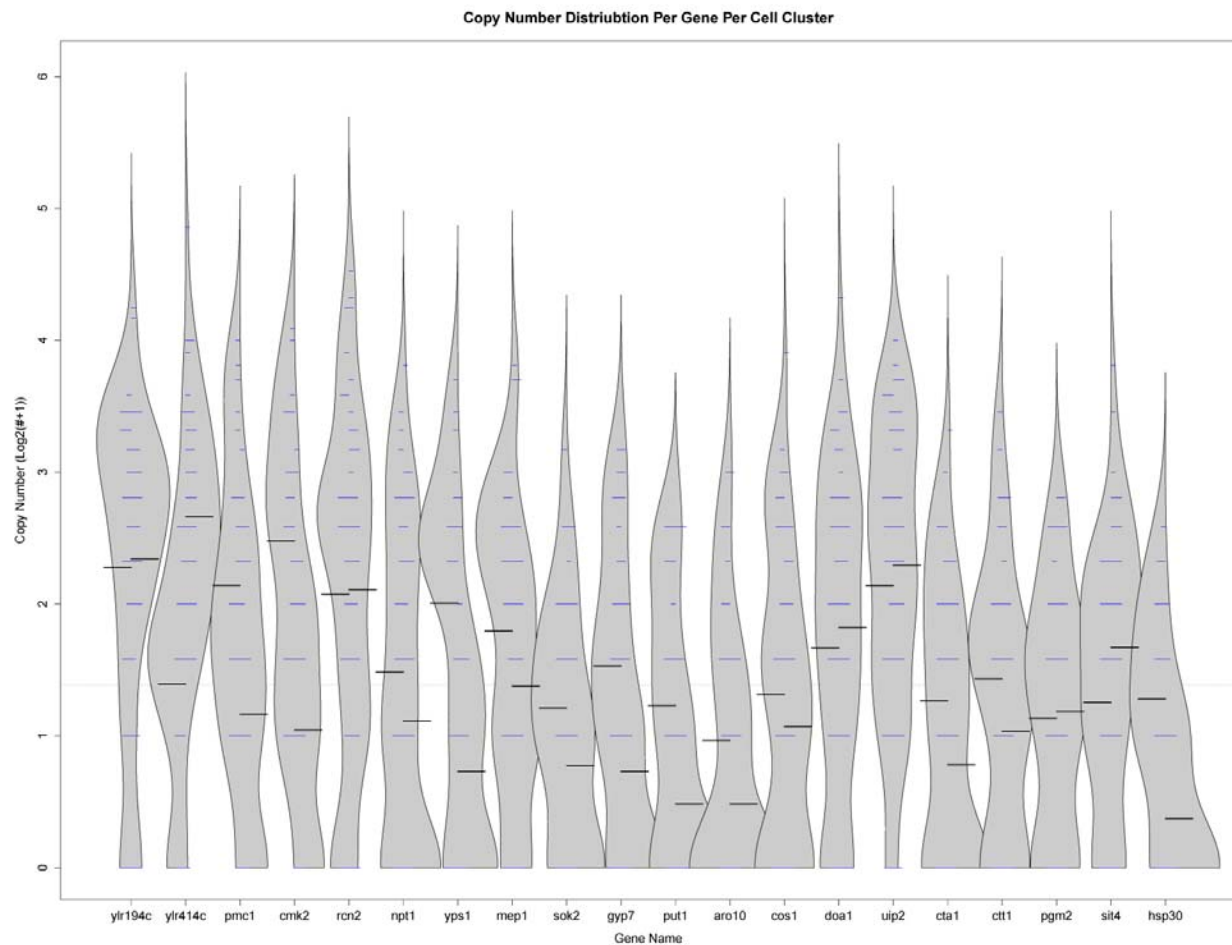
Supplementary Figure 10. Barcode crosstalk measurements. A 3 color barcode is hybridized and imaged. The leakage of that barcode into other barcodes is shown on the histogram, representing the errors in detection and analysis. A total of 20 cells are counted in each case. A) A barcode with Cy5 emitters and all 3 activators, hybridized against *PUN1*. B) The worst case scenario, with Cy3 activators and all emitters hybridized against *YPS1* which is present at lower abundances than *PUN1*. Because Cy3 can be activated by 405 and 473nm lasers, there is more crosstalk into those channels. We observe that there is a relative low level but uniform background of barcodes observed due to autofluorescence in the cells and nonspecific blinking events. This background is additive to the barcode quantitation and does not scale with the copy number of the genes. C-D. Single dye pair crosstalk ratios. 12 probe pairs are hybridized against *PUN1* coupled with each combination of fluorophores. Then the false activation rate in different STORM channels are measured for Cy5 (C) and A750 (D) emitters. Crosstalk from Cy3-Cy5 into Cy3-A680 is 11.6% and negligible in the reverse direction.



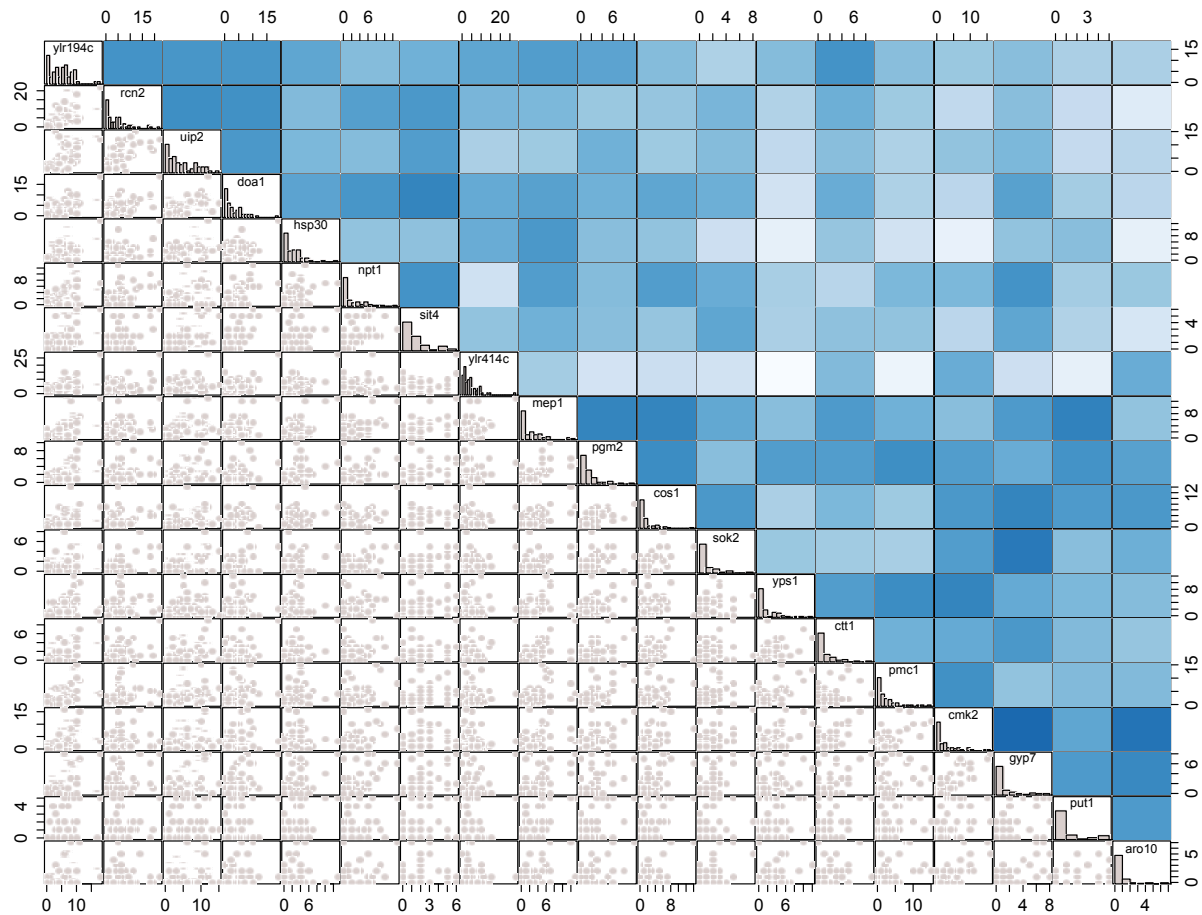
Supplementary Figure 11. Imaging Scheme. For each activator we sequentially went through one cycle consisting of looping the pattern of imaging frames followed by blank frames 20 times. We followed this pattern by imaging just Cy5 for two blank frames every 5 frames until cy5 was completely exhausted after 30 additional frames.



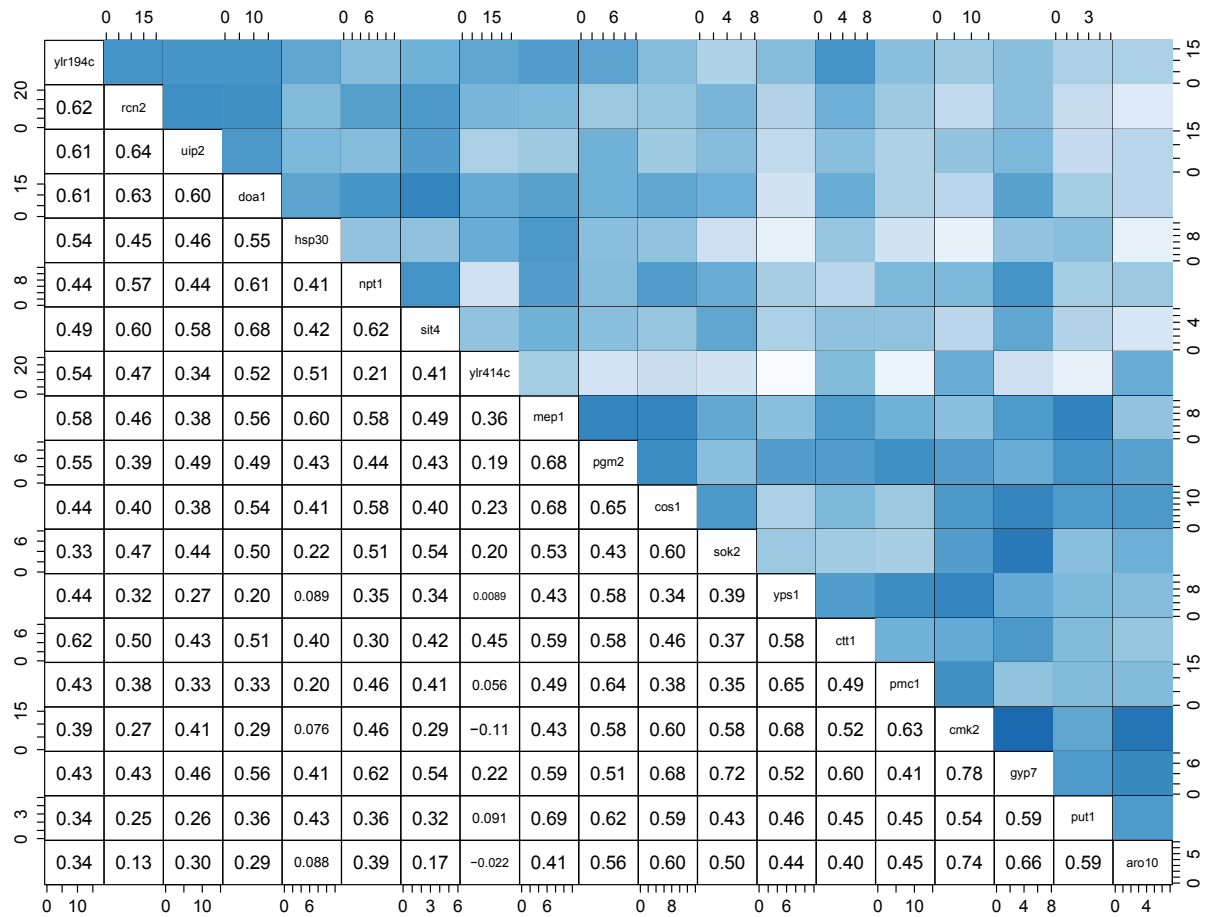
Supplementary Figure 12. Statistical analysis of barcode scramble experiment. Two outliers are present with cooks distances greater than 1 that greatly skew the correlation. The first point was dropped for our regression as removing it placed point 4 back into a tight regression with the remaining data.



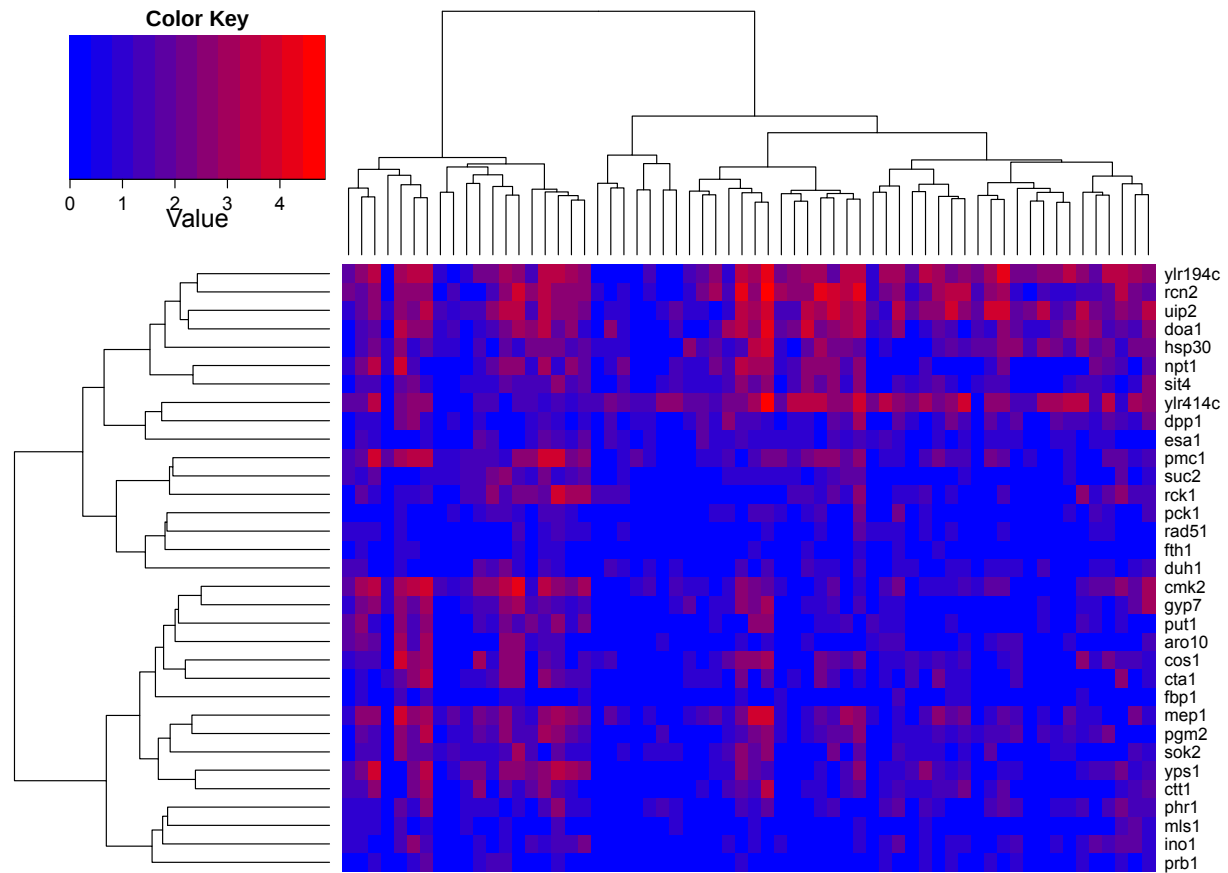
Supplementary Figure 13. Distribution of expression levels for each Crz1 and Msn2 gene. Expression levels are shown in log2 value Bean plots. For each gene, two distributions are shown. On the left are the single cell expression profiles in cell cluster 2: cells with only the combinatorial targets active. On the right are the distributions for that gene in cell cluster 1: cells with all Crz1 target genes on. Black lines indicate the mean values of the distribution. Blue lines mark the integer number of transcripts.



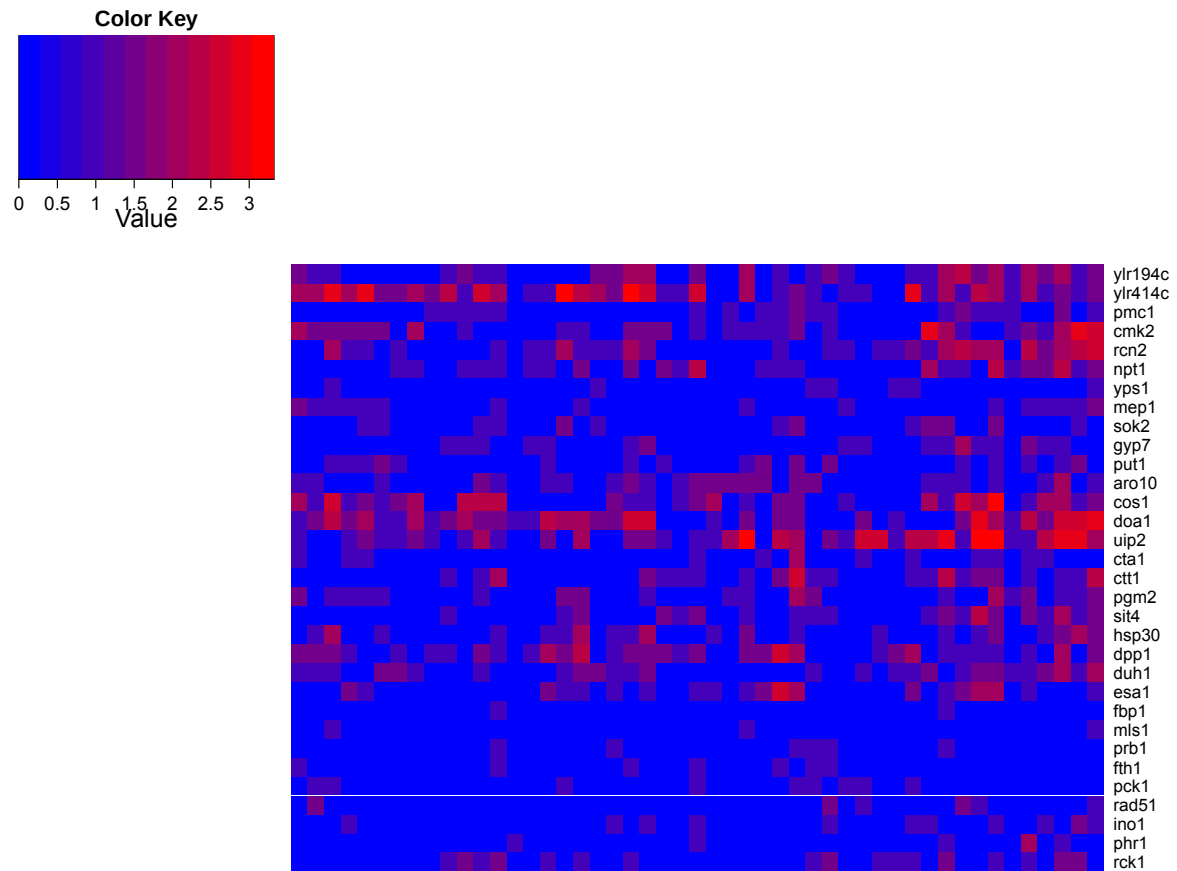
Supplementary Figure 14. WT pairwise gene correlations. Genes are organized in the same order as Fig 4. The gene name and a histogram of each genes copy # distribution is shown in the diagonal. The lower triangle displays the raw pairwise correlation data, while the darkness of the upper triangular quadrant corresponds to the correlation coefficient of each pair.



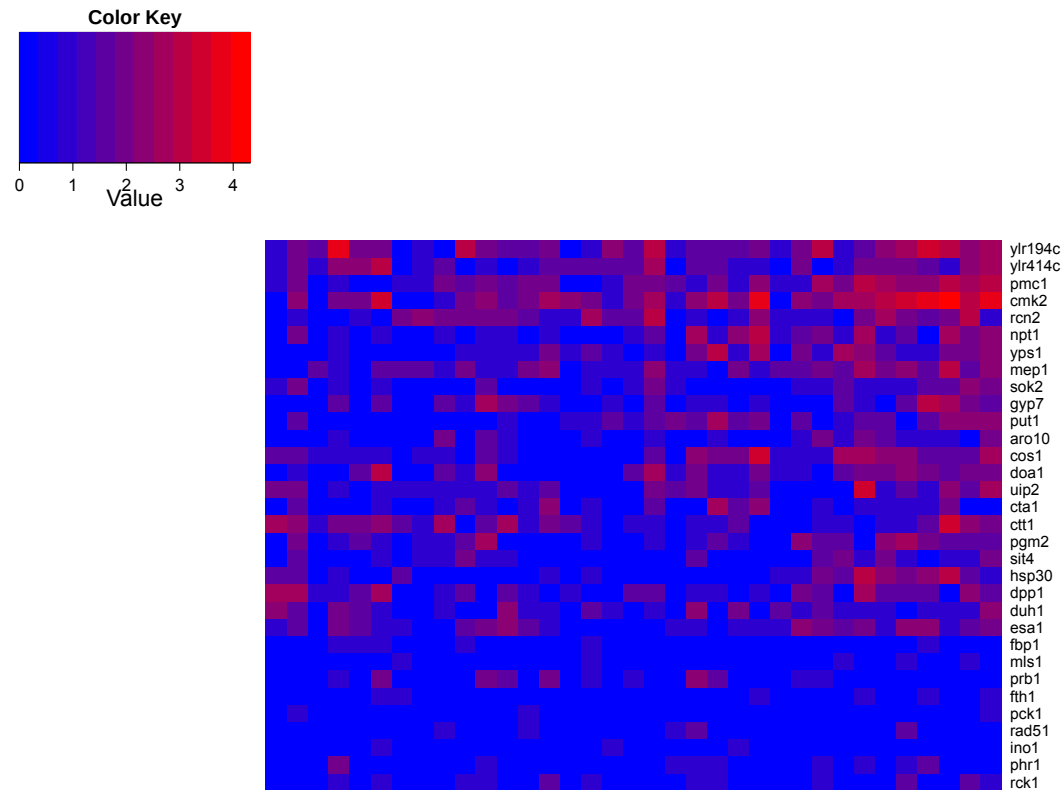
Supplementary Figure 15. Numeric reference for shaded WT correlations shown in **Supplementary Figure 14**.



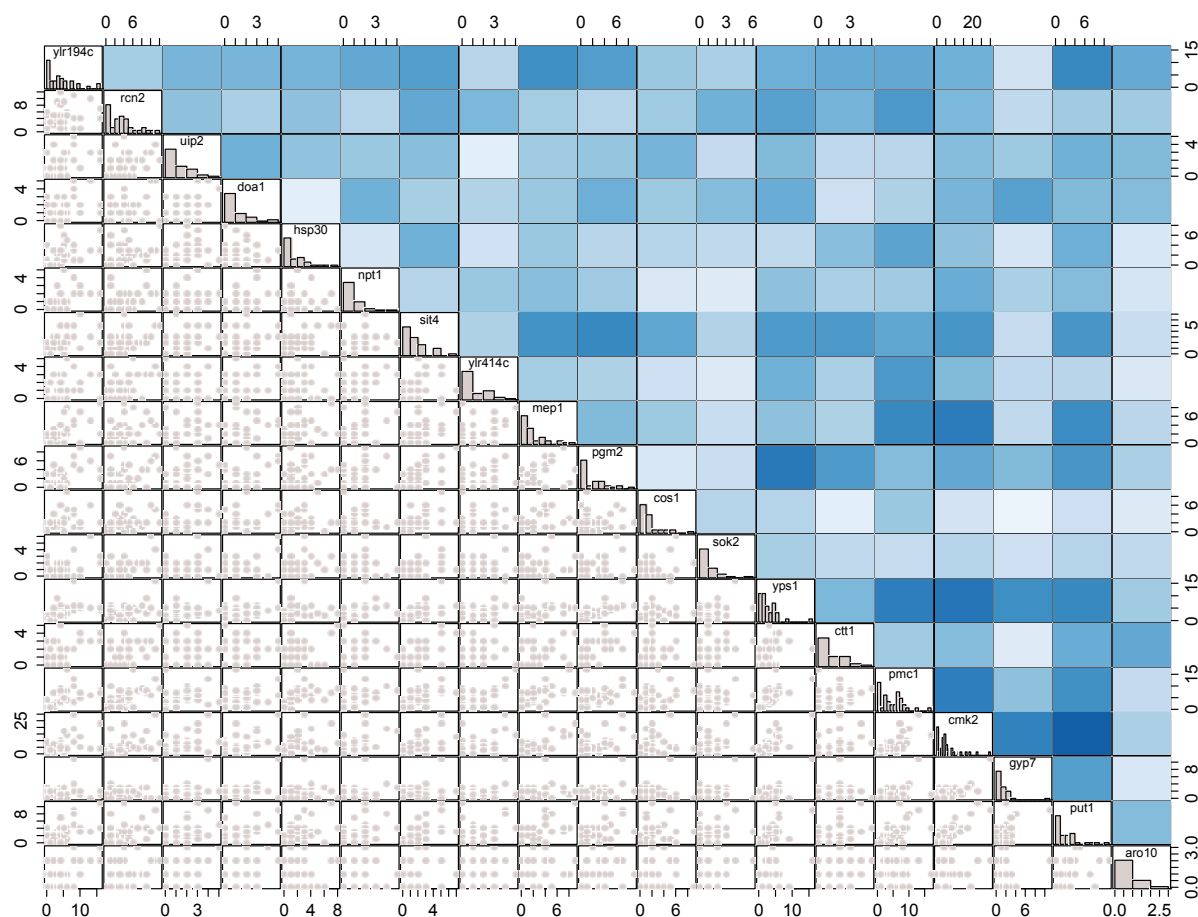
Supplementary Figure 16. WT cell clustering with the aging genes included. The same two clusters of pure and combinatorial Crz1 target genes are preserved. Aging and stress genes roughly fall into 2 separate clusters, apart from the Crz1 and Msn2 genes. Column numbers represent single cell



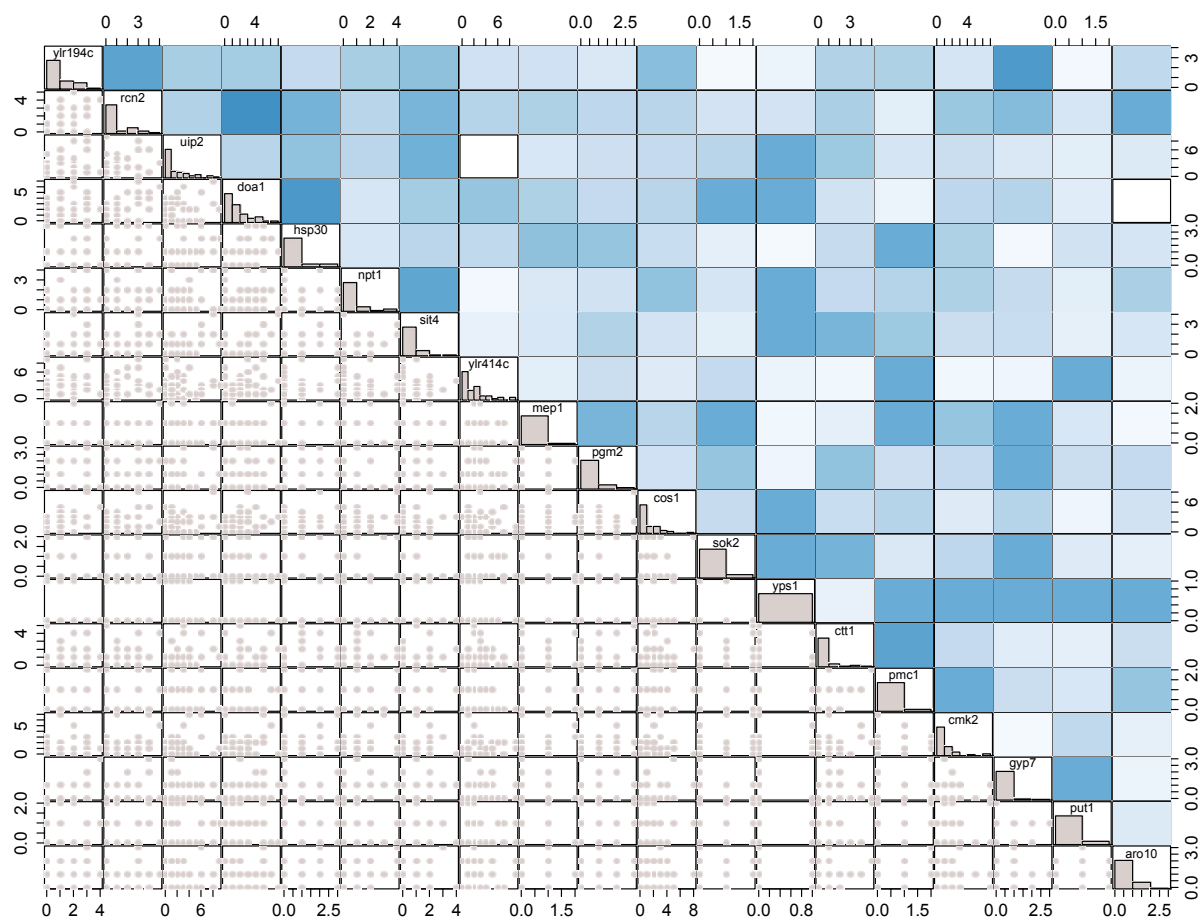
Supplementary Figure 17. Heat maps of single cell gene expression levels under FK506 treatment. The cells are treated with 50mM CaCl_2 the combinatorial genes are plotted in the upper half of the figure. Cells are plotted in columns. Combinatorial targets are active while the pure Crz1 targets are inactive, indicating non-Crz1 inputs can drive combinatorial target expression.



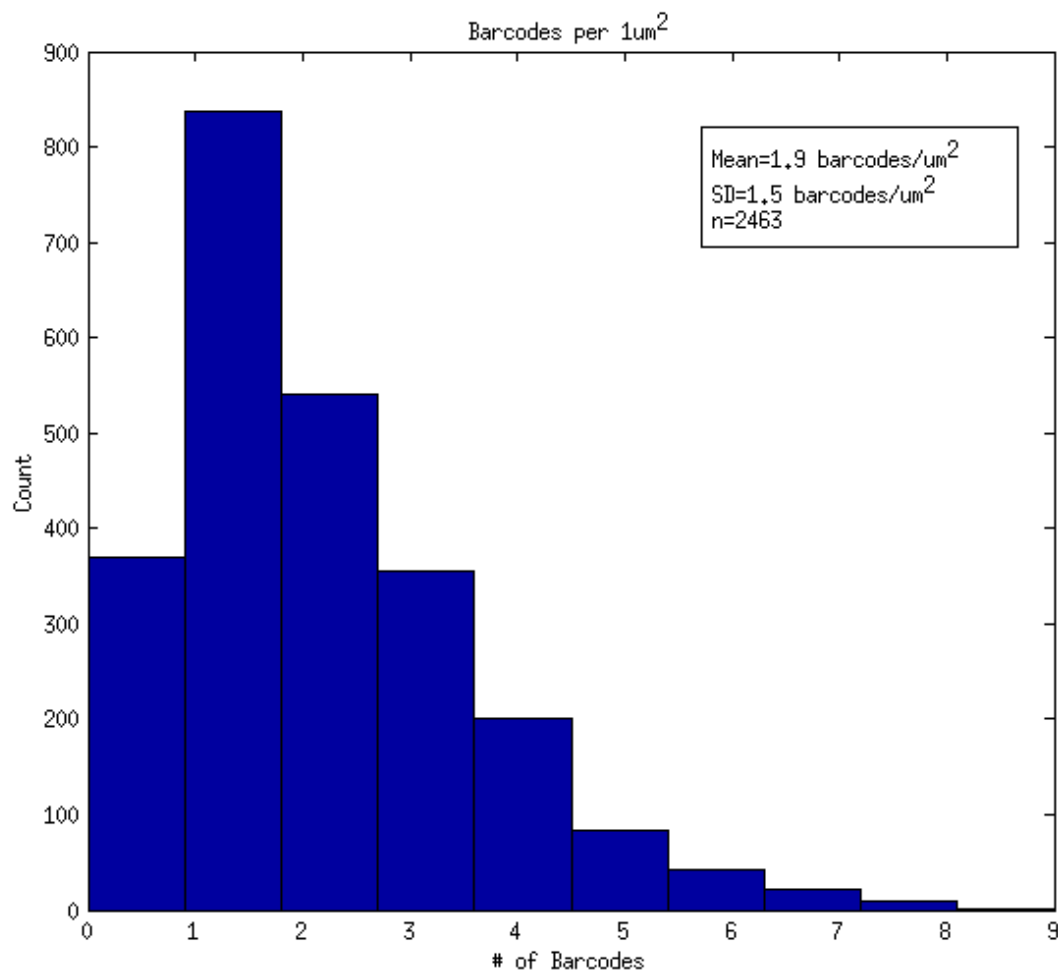
Supplementary Figure 18. Heat maps of single cell gene expression levels in Msn2⁻ Msn4⁻ cells. The cells are treated with 50mM CaCl₂ the combinatorial genes are plotted in the upper half of the figure. Cells are plotted in columns. Most cells show coordinated expression among Crz1 and combinatorial target genes, suggesting Msn2/4 was the major factor in driving heterogeneous expression of combinatorial genes from the pure Crz1 genes.



Supplementary Figure 19. Msn2⁻ Msn4⁻ pairwise gene correlations. Genes are organized in the same order as Fig 4. The gene name and a histogram of each genes copy # distribution is shown in the diagonal. The lower triangle displays the raw pairwise correlation data, while the darkness of the upper triangular quadrant corresponds to the correlation coefficient of each pair. Note the overall smaller difference in correlations between clusters of genes compared to the WT plot.



Supplementary Figure 20. FK506 pairwise gene correlations. Genes are organized in the same order as Fig 4. The gene name and a histogram of each genes copy # distribution is shown in the diagonal. The lower triangle displays the raw pairwise correlation data, while the darkness of the upper triangular quadrant corresponds to the correlation coefficient of each pair. Note the overall low levels of expression.



Supplementary Figure 21. Barcodes density in the current 32 gene multiplex. A histogram of the number of barcodes found per $1\mu\text{m}^2$ region is shown. The overall distribution is approximately poissonian ($\sigma^2 / \text{mean} = 1.3$), indicating a random configuration for the 32 profiled genes. On average, barcodes were relatively sparse at 1.9 barcodes per μm^2 . Nonetheless, certain regions had higher densities, necessitating the use of SRM barcoding. The low overall density of barcodes enables barcoding to be scaled to much higher numbers. Less than 2% of the cell was occupied by barcodes as determined from the average barcode size of $(100\text{ nm})^2$ and the mean barcode density (this figure).

Crz1 Genes				Aging and stress genes		
YLR414c	123			cta1	179	1- 405 cy5
YLR194c	239			dpp1	125	2- 488 Cy5
cmk2	789			duh1	795	3- Cy3 Cy5
pmc1	389			esa1	895	5- Cy3 Cy5
cos1	127			fbp1	157	7- 405 Cy7
mep1	289			fth1	158	8- 488 Cy7
npt1	189			ino1	159	9- Cy3 Cy7
put1	378			m1s1	257	
yps1	379			pck1	258	
sok2	279			phr1	259	
gyp7	278			prb1	785	
aro10	178			rad51	358	
doa1	128			rck1	359	
rcn2	139					
Msn2 genes						
ctt1	137					
hsp30	238					
pgm2	237					
sit4	138					
uip2	129					

Supplementary Table 1. Barcode Assignments for the 32 genes profiled. Codes 135, 235, and 895 are left empty. In the barcode scramble experiments, the activators are permuted. 1->2, 2->3, 3->1.

Gene	Barcode
pun1/YLR414c	123
YLR194c	137
cmk2	789
pmc1	179
cos1	238
mep1	379
npt1	279
put1	189
yps1	178
sok2	378
gyp7	389
ao10	289
doa1	239
rcn2	127
ctt1	128
hsp30	139
pgm2	138
sit4	129
uip2	237
cta1	278

Supplementary Table 2. Circularly permuted barcodes used in switched barcode experiment (**Figure 3** and **Supplementary Figure 12**). Barcodes are permuted following the pattern: (405->488, 488->cy3, cy3->405).

gatctcacgctacaccatagaatgaa	ylr414c-1
catcaaaccctggtagttcctaccaa	ylr414c-2
tatgctttaggatgtatttgatgtat	ylr414c-3
actaatagggcggcaaaggcgaaaaa	ylr414c-4
ccttatgtggatgatccagcgcaata	ylr414c-5
caataccaataagaatggtaatgaac	ylr414c-6
attttacttttagtttttcgggcaa	ylr414c-7
cagagcctcattgttggatattgt	ylr414c-8
ggataccgtgaggcgaagaacatgat	ylr414c-9
tacgaccaaagccctatatttatata	ylr414c-10
agaactcaaagaaggagcaccgtcg	ylr414c-11
cacagtaaattttatttatgggactg	ylr414c-12
acggacgctaccttaccgttgactg	ylr194c-1
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gcagttgaagttgtgcttacggcag	ylr194c-7
tgctggtggtttgccttgtgcatcc	ylr194c-8
cataggtgttgctgacgacgttgct	ylr194c-9
acagttgatgcgctttcttgggctt	ylr194c-10
gtttgagctttcctttgtgagcta	ylr194c-11
agtctttttgagcagcggctagagt	ylr194c-12
atgcagacttcaatttcatttgctc	cmk2-1
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cgcaaagaaaaccctttcttaacgt	cmk2-5
tgaagtaatccatggatcgtccagc	cmk2-6
tcaatctcaatgccttcaagatgaa	cmk2-7
tatggcatatggaaggtaccgggt	cmk2-8
ttcaacgctttcggcaataaaaagga	cmk2-9
caccaatggaccatatacacaaagg	cmk2-10
ggtgccacataaccaacgatccgg	cmk2-11
caattgttagctataccgaagtcc	cmk2-12

Supplementary Table 3. Oligo probes for smFISH. 5' amine modified.

Cmk2	F TCG CCT CTG GTA ATT GCG GAC
Cmk2	R TAA CCC AAC GAT CCG GCT GC
Pmc1	F TTG TTG CGG TCA CTG GCG AT
Pmc1	R AAG CCT CTC TGG CAA CCT CC
Ylr414c	F GCT ACG CTA TCT TCG TTG GGC
Ylr414c	R CTG GAT ACC GTG AGG CGA AGA
Ylr194c	F AGC AAC TCT GCC GTA AGC ACA
Ylr194c	R GTC GTT GAG GAG GAT GCG GA
Npt1	F GGG AGA TCC TGC CAC TGT GA
Npt1	R AGG TCC ATC TGT GCG CTT CG
Gyp7	F ACG ATG GGA GGC TGA GGG TC
Gyp7	R ACC CCA AAC TTT CCC TCG CA
Put1	F GGCGATAAAACGGGCACTGA
Put1	R AGGCGACAACCAAGTGACCAA
Yps1	F TTGACGGGAACGGGCAGTG
Yps1	R CCGAAGCAGGCACGGATTGA
Actin	F ACG TTT CCA TCC AAG CCG T
Actin	R GGA ACG ACG TGA GTA ACA CCA

Supplementary Table 4. qPCR Primers

Supplementary Note

Hybridization efficiency

To determine the hybridization efficiency of the probes, we used photobleaching to measure the number of bound probes. Twelve 27mer probes targeting *CMK2* were coupled to Cy3 and imaged with a 532 nm laser. Discrete photobleaching steps were observed corresponding to bleaching of single fluorophores (**Supplementary Figure 1**). The average step size was ~300 cts. Using this value as the average fluorophore intensity, we estimated the number of probes bound per mRNA based on the dot intensities in the image before photobleaching. Some variations in intensity are likely due to unevenness in illumination and homo-quenching effects of closely spaced fluorophores. We found that on average 8.1 probes are bound out of the total of 12 probes, suggesting a hybridization efficiency of $67.5 \pm 9.1\%$ (**Supplementary Figure 1**) per probe. The observed distribution is consistent with a binomial distribution with the probability of each probe binding at 67%. Less than perfect hybridization efficiency may be due to the tertiary structure of the mRNA molecule and heterogeneities in bound ligands such as proteins on the mRNA. We demonstrate a more robust spectral coding scheme in which single-colored probes are distributed throughout the mRNA. If occluding molecules are bound to a small region of mRNA, they will only block a subset of the probes in every color, as opposed to removing a single color completely.

This hybridization efficiency implies that mRNAs tagged with 4 probes have a 99% chance of being detected with at least 1 probe bound. This is consistent with our observation that $96 \pm 2\%$ ($n=29$) of molecules spatially overlapped in all three channels in the 3 color *PUN1* probes.

3 color STORM spatial reconstructions.

For the 3 color STORM *PUN1* centroid reconstruction, we observed that $74 \pm 8\%$ ($n=28$) of codes reconstruct correctly. The photoswitching dye pairs improve background rejection, as both probes are required for the fluorophore to be re-activated, therefore non-specifically bound emitter probes in the cell cannot be reactivated. In comparison, directly labeling oligos with cyanine dye covalently-linked pairs will have the same non-specific background as standard FISH and have drastically increased blinking rate¹ due to the complex photophysical properties of the cyanine heterodimer. Indeed, we observed prior to inactivation, cells contained a hazy background of singly bound probes in addition to the hybridized FISH spots. After imaging for 4-5 frames, these non-specifically bound molecules switched off and blinked at their respective non-specific activation rate of cy5. Upon activation with 405,473 or 532nm lasers, these background probes did not reactivate. It is highly unlikely that probes with an activator would be bound close enough (<1 nm) to activate an emitter dye. The majority of spots that reactivated were specific mRNA targets, although noise was observed from cellular auto-fluorescence and probe complexes. Some of this noise was due to x-talk among specifically bound dyes. We observed x-talk ratios of around 7% for the most egregious Cy5- dye pairs (**Supplementary Figure 10**). To ensure proper coding fidelity we only selected identified barcode colors that activated at least 3 standard deviations above the x-talk ratios. It has been previously reported that 473nm laser can

activate cy3-cy5 pairs with 10-20% efficiency. We adjusted the 473nm laser power such that it is higher than the non-specific blinking rate, but less than the power needed to consistently activate Cy3 Cy5 probe pairs.

The cost of this background rejection of using probe pairs is reduced effective hybridization rate. As both probes are required for a functional dye pair, the effective hybridization efficiency is $(67\%)^2=45\%$. Thus the probability of having at least 1 pair formed out of a redundant set of 4 probe pairs is $1-(0.45)^4=0.9$. With a 3 color barcode, the theoretical probability of having all three colors present is $0.9^3=0.72$. We observed a $61\pm8\%$ probability (50 out of 85 reconstructions) that 3 colors were present on a given mRNA, and a $33\pm6\%$ probability (28/85) of resolving only 2 colors. We observed no differences in hybridization efficiency between different mRNAs, and the relative ratios between average expression level were recapitulated in our qPCR and smFISH experiments (**Figure 4a,b**). This data suggests that our STORM results accurately capture the relative ratios of mRNA expression, and can be much improved through increased probe redundancy.

The effective hybridization efficiency can be improved by using more probe pairs. With 8 probe pairs per position, the 3 color spatial coincidence rate is increased to $>95\%$. The long term solution is the development of super-resolution fluorophores with improved contrast ratios. With reduced blinking, fluorophores can be directly labeled to oligos. As in the experiments, only 4 redundant probes are needed to co-localize in 3 colors with 96% probability and 6 colors with 93% probability.

Our use of physical compression allowed us to image most RNAs in a single focal plane. This simple physical treatment permitted us to forgo axial resolution of barcodes. Currently, 2 approaches are available to improve axial resolution. Some approaches, such as dumbbell shaped point-spread-function², can improve axial resolution down to 50 nm. Such a resolution is sufficient to resolve overlapping molecules, but won't be high enough to resolve discrete barcode regions on a single RNA. Interferometric PALM³ and dual objective astigmatic STORM⁴ would allow us to reach axial resolutions below 20 nm. Such a high resolution would allow us to accurately capture the 3D structure of barcodes, dramatically improving the fidelity of spatial barcoding.

Scaling up multiplexing capacity with spatial and spectral barcoding

Spatial and spectral barcoding schemes have different strategies for scaling up their throughput. Spatial barcoding is efficient. In principle, five-position barcodes allow $9^5/2= 29525$ genes to be tagged simultaneously in single cells. In practice, super-resolution barcode readout accuracy and labeling density are constrained by the non-specific 'blinking' of the Cy5 dyes (i.e. contrast ratio), occurring at 1 in 200 frames per molecule⁵. In addition, spatial barcoding requires mRNAs to be stretched out to resolve the spatial sequence of colors. We experimented with different fixation conditions and methods to extend mRNAs, but found compressing cells to be the best method for consistently stretching out transcripts. As thick sample are routinely squeezed to reduce optical sectioning for FISH imaging⁶, spatial barcodes may be readily resolved in compact and compressible systems such as embryos.

However, not all biological samples can be compressed, such as tissue samples or biofilms. Spectral coding provides an alternative labeling scheme suitable for such experiments. In this scheme, we note

that the multiplex capacity increases exponentially with the number of fluorophores available. In principle, cyanine dyes can be extended further into the infrared region to act as additional emitters. Recent works⁷ suggest that 1 additional emitter is already available, allowing potentially close to a thousand ($_{12}C_5=792$) genes to be multiplexed.

Terminology used to describe STORM Imaging

Throughout the paper we refer to three sources of STORM activation, specific, non-specific and false. We have used this terminology as it provides a direct analogy to potential sources of error in our imaging routine. Specific activation refers to the desired activation of the intended dye by excitation of the activator dye. Non-specific activation refers to excitation of the emitting dye due to absorption of the imaging light, often referred to as blinking. False activation refers to activation of the emitter due to absorption of the wrong wavelength by the activator dye.

Crosstalk and accuracy of the barcode readout.

The spectral coding approach is more robust because errors associated with identifying spatial positions can be avoided. However, crosstalk among different fluorophores can impede the identification of the proper barcode and result in leakage amongst the barcodes. To control for crosstalk, we performed several control experiments.

First, we imaged individual dye pairs with the full imaging routine, going through all activators and emitters, to quantify the amount of leakage from each dye pair into the others. By examining all 7 dye pairs used in our study, we found the most leakage occurs from Cy3 activators, which can be activated by the 473 and 405 nm lasers. However, Alexa 488 and Alexa 405 cannot be activated with the 555 nm laser, so the crosstalk only appears in one direction. From the single dye pair experiments we quantified the idealized x-talk with 12 of the target dye pairs, imaged in exactly the same routine as our barcode quantification. There is a small amount (~1%) of non-specific activation in Alexa 405 and Alexa 488 with 556 nm activation, due to non-specific blinking of the dye pair⁶. The probe pairs that can be x-talked to, A488 and Cy3, X-talk at a higher rate than previously reported (6-6.5%) (**Supplementary Figure 10**). This may be due to the close proximity of the dyes to each other in our probe design. This x-talk was still clearly separable from signal. For A680, we only used the cy3-A680 pair, thus no crosstalk between A680 dyes can occur. To reject the false activation of cy3 by the 473 nm laser, we discard activations in the 473 channel that are less than 30% of the activations observed in the 555 nm laser channel. Similarly, we set the threshold for rejection at 15% and 30% respectively for 405 nm activation of A488 and Cy3. In addition, there is crosstalk between the cy5 and A680 emitter channels. Since this crosstalk only occurs in the cy3 activation channel, we compare the activation intensity in cy3-cy5 vs cy3-A680 channels and found about 12% crosstalk between the 2 emitters. Thus, any activation in A680 that is less than 30% of the intensity in cy5 is rejected. All of these thresholds are more than 3 SDs from the measured cross-talk values (**Supplementary Figure 10**). We confirmed that the tertiary structure of mRNA did not result in any probe crosstalk. We observed no false activation from probe positions not directly proximal to the emitter probes, in agreement with the photoswitching distance of ~1 nm.

Second, to test the accuracy of the 3 color barcode readout, we used the barcode that is the most prone to crosstalk, which is the cy3 activator paired with all three emitters. This probe set against *YPS1* is hybridized and the correct barcode is picked up, while several barcodes are also observed at a 20% crosstalk rate. However, this represents the worst-case scenario for crosstalk, since Cy3 can be activated by both 405 and 473nm lasers. In addition, the gene targeted with this probe set is relatively low copy number, so false barcodes due to cellular background and nonspecific blinking appear at relatively higher frequency compared to the correct barcode (**Supplementary Figure 10**). A different 3-color barcode with 405, 488 and cy3 as activator and cy5 as the emitter, shows a much lower crosstalk ratio (**Supplementary Figure 10**). Most of the extraneous barcodes observed are due to background blinking in the cell and do not scale with the copy number of the genes probed. Thus, there is a constant background of barcodes that is additive but not multiplicative to the real barcodes.

Third, when we analyzed data for the full dataset with 32 genes, we examined the frequency of observing the barcode position that was not coded. With a total of 35 possible coding positions in our current scheme, there were 3 empty code positions which should not show up. The mean false identification frequency was 0.67 ± 0.84 copies per cell, suggesting our entire barcode set imaged simultaneously is not significantly affected by false positives. In addition, we performed analysis on the full data set with a single gene barcode dropped out, and we observe that the empty position which is normally present at 4.9 ± 2.3 copies per cell is now present at 0.75 ± 0.84 copies per cell, indicating small amounts crosstalk into that position from other barcodes.

Fourth, we took a 20 gene probe set containing cy5 and A750 emitters, and circularly permuted the activators (405->488, 488->cy3, cy3->405). This effectively scrambled the barcode assignment since the emitters remained in the same position. We observed a strong correlation between genes measured amongst both probe sets, indicating no significant bias is introduced by a particular assignment of the barcode (**Figure 3**). One significant outlier existed in our analysis, *YLR194C*. This outlier was dropped based upon its high Cook's distance of 2.08226 (**Supplementary Figure 1**). A regression with an R^2 value of 0.88 was obtained following removal of the one outlier connoted in red. The other large outlier with a high copy number also has a high cook's distance of 3.5515. Despite its leverage, the point fits well on a regression where both of the aforementioned points are dropped, so it was retained in our analysis.

Fifth, we performed single molecule FISH experiments measuring the expression of 11 genes, including 8 *crz1*, 1 *Msn2* and 2 aging genes. We observed a R^2 of 0.95 between the mean levels measured by smFISH and the barcode approach (**Figure 4a**).

Sixth, we also performed q-PCR experiments measuring the mean copy number of 8 *crz1* target genes. We observed a correlation of 0.95 between the qPCR and the barcode data. The qPCR experiments were performed in triplicates and quantitated using 1x, 10x and 100x serial dilutions (**Figure 4b**). The PCR amplification efficiency for each gene was determined from linear fitting of serial dilutions.

Spatial capacity of super-resolution barcoding

The size of mRNAs that we reported is approximately 100 nm (**Supplementary Figures 2-4 and 5-9**). At this size, a typical cell of $(10 \mu\text{m})^3$ can fit in about 10^6 of these $(100\text{nm})^3$ volume elements, sufficient

to accommodate a significant fraction of the transcriptome simultaneously in the cell. In the reconstruction shown in **Supplementary Figure 9** it is apparent that there is at least 20x more space left to accommodate additional barcodes. With the implementation of 3D storm, there will be 10x more room in the axial direction. In mammalian cells, the much larger available space, 20-50x larger than yeast, will compensate for the higher transcript levels (also 20-50 fold).

These calculations do depend on the assumption that transcripts are homogeneously distributed throughout the cell. In our observations, and in that of other authors using smFISH in eukaryotes, a homogeneous spatial distribution of RNAs outside of the nucleus is usually observed (**Supplementary Figure 21**). If certain transcripts are found to cluster, they can be removed from the large dataset and analyzed separately. We imagine such a combination of techniques should cover the vast bulk of *in situ* transcriptional profiling experiments.

In the case of the nucleus, we and other authors have found dense transcriptional sites to be rare in FISH experiments. In yeast, we found transcriptional sites to be in <5% of cells at any given time. Given the density of very highly expressed genes and the large size of the mammalian nucleus, we expect that most of these sites can still be identified and barcoded using our technology. In the cases where the nuclear density is too high to distinguish the transcripts of interest, we can still use the intensity ratio to infer the most likely barcode and assign its abundance to transcriptional active sites.

In addition, a recent paper from the Tyagi group⁸ showed that mRNAs in neurons are packed singly into transport granules separated by at least 200nm, rather than compacted in dense granules as previously thought. While non-uniform distribution of mRNAs in cells could in principle pose a challenge to the barcode readout, few existing smFISH experiments in eukaryotes suggest that the spatial distribution of transcripts will be a problem.

In our current 32 gene multiplex experiment, we need super-resolution imaging to spatially separate transcripts, as shown in **Supplementary Figure 9**. In this figure, the axis units are pixels, which on our setup correspond to 130nm. A diffraction limited spot would span 6-7 pixels end-to-end. One can see that the average RNA is confined within 1 pixel. Many mRNAs reside within one diffraction limited region, making it impossible to resolve many neighboring barcodes without super-resolution microscopy.

Our single cell profiles cover a wide gamut of copy #'s, demonstrating the wide dynamic range of SRM barcoding. Several of the 32 genes we profiled are highly expressed, on the level of major structural proteins. For example, *CMK2* is expressed at 7500 copies of proteins compared to 5000-10k copies for tubulin, as measured using quantitative westers and GFP⁹. Many other genes, such as *PUN1* and *YLR194c* are expressed at similar levels (**Supplementary Figure 13**). The average mRNA copy number might seem low because expression is bursty and heterogeneous among cells, but is often not low in individual cells. In our experiments *CMK2*, *PUN1* and *YLR194C* were additionally induced by extracellular calcium, so we expected the protein copy # to be well above these reference measurements.

The available space should be more than sufficient to accommodate ~1000 genes at a time. We imagine the typical use case of single cell barcoding will follow-up traditional high-throughput techniques such as

RNA-seq, so many uninteresting highly expressed housekeeping genes will not be probed in the barcoding experiment. By measuring highly-multiplexed gene expression *in situ* with spatial information, our technique will make a significant contribution to systems biology not possible with existing technologies.

Single Cell Profiling and Correlation

We measured the copy number of probed genes by tabulating the barcode reconstructions in single cells (barcodes in Table S1). We did so for 62 cells. On average 2-3 cells were observed per field of view. Each set of STORM images took ~10 minutes to acquire; the imaging schematic is shown in **Supplementary Figure 11**. We manually found the positions of 10-15 cells and set up an automated stage to scan through the positions for STORM imaging. It takes approximately 5-6 hours to collect 60 cells. The field of view (FOV) was kept small to reduce auto-fluorescent background from glass. In principle, quartz slides can decrease background and allow the FOV to expand, dramatically increasing throughput.

Msn2 has a protein paralog, Msn4 that largely binds the same promoters and pulses synchronously with Msn2. Throughout the text, when we refer to Msn2 pulses, we are referring to the combined effect of both TFs

Additional discussions of analysis protocol

For this proof-of-principle we performed a simple analysis that gridded the data into a fixed width to initially find barcodes. After barcodes were found, the grid was removed and a local center of mass calculation was used to find the center of a barcode cluster. A fixed grid size of 184 nm was used for simplicity, since most barcodes fall within a 100 nm diameter. In the future, more intelligent clustering algorithms will be needed to identify barcode clusters. We note that this is a well-studied problem in computational image analysis and a variety of tools are available to address this problem, but they are beyond the scope of the current paper.

In our current analysis protocol, we do not exclude multiple activation events since they are infrequent in our movies and largely constitute emission from a single RNA. We are able to use more of the photons collected in the experiment to determine barcodes more accurately. However, for denser samples, a more stringent application of thresholding, or an application of the ideas in the photobleaching approach^{10,11} and fluctuation imaging (SOFI) approach¹² can be implemented for more efficient reconstructions with minimum information loss. These approaches do not require single activations, but rather use intensity fluctuations for super-resolution imaging. Incorporating them in our future analysis protocol will result in more efficient utilization of photons and better barcode reconstructions. Although these methods are not yet implemented, there is no fundamental limitation to incorporating them. Our current protocol was optimized to resolve a multiplex of 32 barcodes from each other, as demonstrated in **Supplementary Figure 9**. We anticipate this protocol will carry over for multiplexing <100 genes. We expect to incorporate the additional changes in the next iteration of barcoding when we go above 100 genes.

Sequences for probes used in the 32 gene multiplex are available upon request.

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