A cross–nearest neighbor/Monte Carlo algorithm for single molecule localization microscopy defines interactions between p53, Mdm2, and MEG3

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Abstract

The functions of long noncoding (lnc)RNAs such as MEG3 are defined by their interactions with other RNAs and proteins. These interactions, in turn, are shaped by their subcellular localization and temporal context. Therefore, it is important to be able to analyze the relationships of lncRNAs while preserving cellular architecture. The ability of MEG3 to suppress cell proliferation led to its recognition as a tumor suppressor. MEG3 has been proposed to activate p53 by disrupting the interaction of p53 with Mdm2. To test this mechanism in the native cellular context, we employed two-color direct stochastic optical reconstruction microscopy (dSTORM), a single-molecule localization microscopy (SMLM) technique to detect and quantify the localizations of p53, Mdm2, and MEG3 in U2OS cells. We developed a new cross–nearest neighbor/Monte Carlo algorithm to quantify the association of these molecules. Proof of concept for our method was obtained by examining the binding between MEG3 and p53, and Mdm2 and p53. In contrast to previous models, our data support a model in which MEG3 modulates p53 independently of the interaction with Mdm2.

Introduction

Long noncoding RNAs (lncRNAs) function in cell-type and subcellular localization–dependent contexts; how they do so is poorly understood. The human MEG3 lncRNA gene is located on chromosome 14q32 and belongs to the conserved, imprinted DLK1-MEG3 locus (1,2). MEG3 transcripts are detected in a wide range of normal tissues, including endocrine tissues, brain, gonads, and placenta (1). MEG3 modulates the activity of multiple miRNAs; for example, MEG3 functions as a decoy for miR-138 (3) allowing it to regulate the generation of IL-1β in macrophages in models of host defense. MEG3 has also been reported to directly interact with DNA to modulate the transcription of TGF-β pathway genes (4).

Based on the observation that MEG3 expression is lost in clinically non-functioning pituitary adenomas, we identified MEG3 as a tumor suppressor (1.5–7). Compared to normal tissue, MEG3 expression is also significantly reduced or absent in meningiomas (8), epithelial ovarian cancer (9), and squamous cell carcinoma of the tongue (10); supporting its role as a tumor suppressor. This function was further supported by studies of tumor xenograft growth in nude mice (11,12). Several studies demonstrated that MEG3 expression causes an increase in cellular tumor antigen p53 (p53, UniprotKB P04637) levels and selectively activates p53 target gene expression (11,13–16), suggesting that MEG3 exerts its cellular functions via p53. However, how the MEG3 lncRNA activates p53 remains elusive. Our previous work indicated that MEG3 functions via activation of tumor suppressor p53 (16). p53 coordinates a transcription program to stall the cell cycle, promote DNA repair, and initiate senescence or apoptosis (17). The primary modulators of p53 activity are the E3 ubiquitin–protein ligase Mdm2 (Mdm2, UniprotKB Q00997) and its heterodimer partner protein Mdm4 (Mdm4, UniprotKB O1515) which constitutively polyubiquitinate p53 for proteasomal degradation, maintaining p53 at low levels (18–20). Thus, modulating the p53–Mdm2/4 interaction is a critical point of regulation for p53 activity. Signal-dependent post-translational modification of p53, including phosphorylation and acetylation, can block Mdm2/4 from binding to p53 and prevent its degradation (21). Stabilization of p53 may also be achieved through interaction with other proteins such as peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) (22,23). It has been shown that MEG3 and p53 can be pulled down in one complex by immunoprecipitation (24,25). Therefore, one possible mechanism for p53 activation by MEG3 is disrupting the p53–Mdm2/4 interaction.

Identifying molecular associations within the spatial context of the cell is necessary to fully define the behavior of MEG3. Single molecule localization microscopy (SMLM) is exceptionally well positioned to provide this information. SMLM is unique from other microscopy approaches in that it provides high-accuracy coordinates of the positions of fluorophores rather than an image (although an image may be reconstructed from
these localizations). As such, SMLM data must be analyzed with very different methods from traditional microscopy data, which are still under active development (26). The first techniques applied traditional fluorescence image analysis approaches to the reconstructed images, although much of the unique information obtained by SMLM is lost this way. More promising approaches have looked at cluster-based and tessellation-based analyses (27,28), enabling the examination of supramolecular assemblies. However, there has been little work done towards using SMLM data to measure single molecular interactions.

To fully understand the interactions of MEG3 with p53 and to test the hypothesis that MEG3 disrupts p53–Mdm2 binding, we developed a new cross–nearest neighbor/Monte Carlo algorithm to quantify the association between molecules from direct stochastic optical reconstruction microscopy (dSTORM) data. We characterized the behavior of this method in silico and demonstrated a proof of concept by examining the binding between MEG3 and p53, and Mdm2 and p53. Future work will build on this technique to examine the relationships of MEG3 to other cell components. In contrast to previous models, our data support a model in which MEG3 modulates p53 independently of Mdm2.

**Results**

Quantifying macromolecular associations by SMLM

We developed an SMLM approach that allowed us to identify potentially interacting molecules by calculating the probability that two localizations were anomalously close. We applied a Monte Carlo
estimation method (Figure 1) that accounts for the local density around a candidate binding pair, partly based on a technique recently introduced for examining the association of sparse mRNAs in neurons (29).

In the first step nearby localizations were grouped into “molecules” using spatial and temporal thresholds (Figure 1A and 1B). A characteristic of dSTORM is that there is no guarantee that a single molecule will be represented by a single localization. Consider the p53 tetramer: it may be bound by up to 4 primary single-epitope antibodies, each of which may be bound by 1 or 2 secondary antibodies, each of which may have 0 to 8 fluorophores attached (despite the average being ~1 dye molecule/antibody), and each fluorophore may blink many times before permanent bleaching. A grouping algorithm is important for dSTORM data to remove such autocorrelated localizations for our downstream analysis, which here assumes that each molecule’s location is independent of each other molecule.

Second, “molecules” from each channel were paired together through an exclusive cross–nearest neighbor algorithm: closest pair identified then removed, repeating until all possible pairs were made (Figure 1B and 1C). The resulting list of pairs is guaranteed to contain all detectable binding events.

The third phase of the algorithm assesses the probability that each pair is associated by chance. Within the local neighborhood (radius $r = 800$ nm, Figure 1D), 10,000 random permutations of the positions of the molecules within this radius were generated and the smallest paired distance measured in each iteration (Figure 1E). The fraction of permutations in which a distance $d_i$ less than or equal to the observed distance $d_{min}$ was recorded as the probability of chance association ($p(chance\ association)$) for that molecule pair (Figure 1F). These steps were repeated for each pair in each cell, and a graph of distance and probability of chance association may be generated (Figure 1G). This plot from a representative cell shows that larger distance is correlated with higher probability of chance association, with wide variability due to local density changes.

Finally, these pairwise measures of association were reduced into a summary value which would correlate with fraction bound. We considered pairs with a probability of chance association less than 0.1 and a distance of less than 200 nm to be bound, and used that value to generate the final output, fraction bound (Figure 1H). In this example, the average distance of the pairs in the “bound” fraction is approximately 50 nm, which corresponds well with the range expected due to the size of the antibody stacks used to detect molecules (up to ~50–70 nm between fluorophores, Figure 2). In this dataset, pairs with a large distance but a low probability of chance association were rare; most of those pairs classified as unbound were due to moderately close pairs in dense areas.

**Algorithm performance improves with lower density, shorter distances**

A natural limitation of our algorithm is that it strongly depends on the density of localizations and the distance between the fluorophores of an associated pair. To characterize this behavior, we simulated 30 distributions of molecules (i.e. the product of the grouping algorithm described above) for each combination of density (100, 200, 500, 1000, 2000, 5000, and 10000 molecules of each kind within a 250 μm$^2$ circle), percent binding (0, 1, 2, 5, 10, 20, 50, and 100%), and physical distance
between fluorophores (10, 20, 50, 100, and 200 nm). Higher density naturally means that the two sets of localizations are closer together on average (Figure 3, columns from left to right), while larger binding distance inflates the value they converge to as more of the molecules are bound together (Figure 3, rows from top to bottom). The Monte Carlo component of the association algorithm adjusts for local density around a putative associated pair, but the algorithm’s sensitivity is reduced by higher global density (Figure 4, columns from left to right). Larger physical distance between fluorophores in a bound pair similarly reduces sensitivity, as the pair becomes harder to distinguish from the background distribution (Figure 4, rows from top to bottom). Normalizing the fraction bound using 0% and 100%
mean values improves the correspondence between true fraction bound and measured fraction bound, but the measured value becomes a severe underestimate of the true fraction bound at higher densities and increased pair distances (Figures S1 and S2). We applied this normalization approach to the biological data to partially correct for global density and distance between fluorophores.

**MEG3–p53 interaction**

We developed U2OS osteosarcoma cell clones containing a doxycycline-inducible MEG3 (U2OS-MEG3) and confirmed that MEG3 was induced 100–200-fold on doxycycline treatment as determined by qRT-PCR. We established conditions for performing dSTORM,
simultaneously imaging RNA using fluorescence in situ hybridization (FISH) and proteins by immunofluorescence (IF). After 20 h induction with doxycycline, cells were fixed and MEG3 was labeled with a tiled probe set conjugated with Quasar 670 (magenta), and p53 was labeled with a secondary antibody conjugated to ATTO 488 (green). Cells were separately labeled for GAPDH mRNA with a tiled probe set conjugated with Quasar 570 (magenta) as a negative control. Large tiled widefield fluorescence images were taken (Figure S3) and ten individual cells were randomly selected from these fields for dSTORM, in each of three replicates. Representative cells are shown in Figure 5, widefield (left three columns) and dSTORM localizations and grouped molecules (right two columns, respectively). The intense fluorescence indicating MEG3 is readily apparent in the nucleus of the cells treated with doxycycline, along with p53, while very little MEG3 is apparent in untreated cells (Figure 5A). Doxycycline treatment had no apparent effect on the GAPDH mRNA distribution (Figure 5B). As seen in the detail of the molecule groups (right column), the grouping operation is slightly biased towards merging nearby clusters.

Using our cross–nearest neighbor/Monte Carlo method, we found a stark difference between MEG3 and GAPDH mRNA in terms of fraction bound (Figure 6A). Two-way nested ANOVA confirmed that the binding fraction was significantly larger for MEG3 than for GAPDH mRNA ($F = 5.487$, $p = 0.04723$, $\omega^2 = 0.06040$), but no significant effect due to doxycycline ($F = 1.740$, $p = 0.2237$, $\omega^2 = 0.009955$) and no interaction effect ($F = 2.670$, $p = 0.1409$, $\omega^2 = 0.02248$). Since only the RNA main effect was significant, a follow-up one-way nested ANOVA was performed within each RNA type. For GAPDH mRNA, ~0.94% of p53–GAPDH mRNA pairs were “bound”, with virtually no change due to doxycycline (-Dox (white box): 0.82% vs. +Dox (gray box): 1.1%; $F = 0.1960$, $p = 0.6808$, $\omega^2 = -0.04127$) and little variability between cells overall, though significant inter-replicate variability was present (blue, red, orange points; $F = 3.781$, $p = 0.008795$, $\omega^2 = 0.2265$) (Figure 6A, right). There are several reasons why the method may have measured a very low level of binding between p53 and GAPDH mRNA. First, p53 is known to have promiscuous non-specific RNA binding capacity (30). Another contributor may be crosstalk due to the spectral overlap of the fluorophore used for GAPDH mRNA and p53.
**Figure 6. MEG3 associated with p53.** MEG3 was induced by treatment of U2OS-MEG3 cells for 20 h with (gray) or without (white) 1 μg/mL doxycycline. The cells were then fixed and stained for 2-color dSTORM of MEG3 and p53 (left) or GAPDH mRNA and p53 (right). For each condition, single molecule localizations were collected from 10 randomly chosen cells in 3 separate experiments. (A) Fraction of pairs bound, as defined by a probability of chance association < 0.1 (i.e., correction for local density) and distance < 200 nm (upper limit for binding distance, accounting for error). (B) Median distance between pairs for each cell (nm). Boxes indicate median +/- upper and lower quartile; whiskers indicate the range excluding outliers. Data points are colored by replicate. * indicates p < 0.05 by nested two-way ANOVA.

In contrast with GAPDH mRNA, the median fraction of MEG3 bound to p53 as defined by this method was substantially higher at ~2.8%, and doxycycline induction did not significantly affect this amount (-Dox (white box): 3.9%, +Dox (gray box): 1.6%; F = 2.496, p = 0.1893, ω² = 0.040281) with wide variability but no significant difference between replicates (blue, red, orange points; F = 1.822, p = 0.1381, ω² = 0.07286) (Figure 6A, left). The lack of effect due to MEG3 induction suggests that the fraction of MEG3 bound to p53 inside cells remains constant across changes in MEG3 expression. For comparison, we also applied a naïve median distance approach, where we calculated the median of the pairwise distances for each cell (Figure 6B). In this simple approach, there was a high degree of overlap between MEG3 and p53 localization change little between conditions. Individual cells were randomly selected from these fields for dSTORM analysis, in each of three replicates. Representative cells are shown in Figure 7, widefield (left three columns) and dSTORM localizations and grouped molecules (right two columns, respectively). Intense nuclear p53 fluorescence is observed on treatment with nutlin-3a, without much apparent change due to doxycycline. Mdm2 levels and localization change little between conditions. Individual cells were randomly selected from these fields for dSTORM (Figure 7).

Using our cross–nearest neighbor/Monte Carlo method, we found that nutlin-3a treatment did not cause a significant change in p53–Mdm2 binding (-Nut: 1.7%, +Nut: 1.7%; F = 0.0002317, p = 0.9882, ω² = -0.006422) (Figure 8A). Doxycycline treatment (MEG3 induction) did not have any significant overall effect (-Dox: 1.4%, +Dox: 1.9%; F = 2.178, p = 0.1782, ω² = 0.007572). There was no significant interaction effect (F = 0.08012, p = 0.7842, ω² = -0.005909). There was no significant inter-replicate variability (F = 0.7478, p = 0.6492, ω² = 0.02600). Importantly, these data suggest that MEG3 does not disrupt overall p53–Mdm2 binding. However, nutlin-3a also did not appear to disrupt p53–Mdm2 binding by this measure. These observations may in part be explained through...
For example, nutlin-3a inserts into the pocket of Mdm2 that binds to p53, preventing Mdm2 from ubiquitinating p53 and marking it for proteasomal destruction \((31)\). Thus, levels of both proteins may accumulate until Nutlin-3a is saturated and excess Mdm2 can bind to p53. This state may be reached with a similar fraction of both proteins bound. It may also be that the interaction is relatively transient, and too close to the limit of detection for this method.

For comparison, we again conducted an analysis based on naïve median distance. As with MEG3–p53 binding, we found this simple approach produced overlapping, hard-to-interpret distributions (Figure 8).

### Discussion

We developed a mathematical approach to analyzing SMLM data that we used to interrogate the interactions of MEG3. Our overall approach takes advantage of high-resolution molecule position data to calculate distances between putative binding partners, assesses the probability that the two molecules are not bound together, and thus provides an overall measure of the fraction of pairs of molecules likely bound together. Using this technique, we distinguished between non-binding pairs (\(\text{GAPDH mRNA–p53}\)) and binding pairs (MEG3–p53) inside cells and quantified the extent
of binding between Mdm2 and p53. The mechanism of MEG3 action suggested by our experiments is different than the previously proposed mechanism in which MEG3 acts by protecting p53 from polyubiquitination by Mdm2. Moreover, the fraction of binding assessed between MEG3 and p53 indicates that there are insufficient stable interactions occurring to effectively inhibit p53–Mdm2 binding. These data suggest that MEG3 activates p53 through alternative mechanisms.

Under MEG3 induction, p53 transcription activation is selective, inducing certain p53 targets (e.g., GDF15) but failing to induce other p53 targets (e.g., CDKNIA) (16). A MEG3–p53 complex may not be competent to induce Mdm2 expression, thereby suppressing the negative feedback regulatory loop. MEG3 also interacts with the chromatin remodeler polychrome repressive complex 2 (PRC2) (32,33), which is responsible for forming heterochromatin at target sites. MEG3 targets PRC2 to certain sites via DNA triplex formation (e.g., TGF-β pathway genes) (4) and protects other sites from PRC2 activity (e.g., MEI locus) (34). A recent investigation of MEG3 structure identified a pseudoknot in MEG3 critical for MEG3-dependent p53 activation, which however was not directly involved in p53 binding (35). It is also possible that MEG3 may modulate the activity of Mdm2 on p53 by forming a ternary complex with them. Similar interactions have been observed with p14ARF (tumor suppressor ARF) (36), UCH-L1 (ubiquitin carboxyl-terminal hydrolase isozyme L1) (37), and the 5S RNP (38). Future work will need to address these alternative mechanisms.

There are some important challenges to the cellular SMLM-based binding analysis we have developed, and which affect SMLM analysis approaches in general. First, despite the 10–20 nm resolution of each localization, the large distance between the molecule of interest and the fluorophore limit the analytical resolution. A typical two-antibody stack can have a displacement of up to ~35 nm from the bound epitope to the conjugated fluorophore; thus, the fluorophores for a bound pair of molecules may be separated by ~50–70 nm or more (Figure 2), depending on the distance between epitopes. In addition, the antibody stack may be free to rotate and flex at the neck, adding variability in the position of the fluorophore during imaging (39). High density also impacts ability to differentiate low fraction binding, as illustrated in Figure 4. These distance issues may be addressed in part using nanobodies as secondary antibodies, which would reduce the distance to ~30–50 nm (Figure 9A). Nanobodies are small single-domain antibody fragments derived from camelids that are emerging as powerful and versatile tools for biology (40-42). Further distance reduction to ~10–30 nm and stable positioning could be achieved by introducing a fusion tag into the target gene and directly binding it with a nanobody (Figure 9B) (40). Further improvements to the algorithm may also be able to address these issues.

A second set of limitations comes from the stochastic nature of SMLM. Fluorophores may blink many times, only once, or not at all (43). This phenomenon makes it difficult to distinguish between nearby molecules of the same type. We employed an aggressive grouping algorithm to address this issue, but the tradeoff is that true separate molecules may be missed. We labeled our own secondary antibodies to control the dye:antibody ratio at ~1:1 to limit multiple blinking, but the labeling creates a distribution and some antibodies will still have multiple fluorophores. Antibodies engineered to have consistent labeling...
stochiometry would be an improvement. A trade-off to limiting the dye:antibody ratio is that many of the secondary antibodies will have no fluorophore, reducing labeling efficiency. SMLM techniques generally have shown a labeling efficiency of at most 60% (26). Improvements to labeling efficiency will enable rare binding interactions to be more readily detected by our method.

A third challenge for our algorithm comes from drift. Autocorrelation drift correction is standard, but it is optimal for defined structures that can be aligned from repeated blinks that occur throughout the acquisition. Singular soluble proteins, which blink only within a small window, pose a challenge for this correction method, and too few blinks overall can prevent the automatic correction from working despite apparent drift by eye. Further, this correction method cannot remove high-frequency variation in position through vibration within the microscope. Fluorescent beads may be used as a fiducial marker at the coverglass surface, but a solution is needed that would work throughout the cell. A sparsely labeled ubiquitous cellular structure, like tubulin, could serve this purpose with an appropriately engineered label. Eliminating drift effects would allow our algorithm to use smaller radii, increasing its sensitivity.

Previously, we found that MEG3 induces p53 stabilization and stimulates p53-dependent transcription activation (16). In this study, we demonstrated that MEG3 lncRNA interacts with p53 inside the cell and can be detected with a novel analytical method using dSTORM. We also demonstrated that the p53–Mdm2 interaction may not be significantly disrupted by MEG3 in cells. Taken together, these data suggest that an alternative mechanism leads to p53 activation. Finally, we believe our binding analysis provides a powerful new tool to assess macromolecular interactions in a native cellular context, with future extensions to 3-dimensional data and multi-protein complexes.

**Experimental procedures**

**Cell lines, media, and growth conditions**

The U2OS osteosarcoma cells (ATCC HTB-96) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco 11995065) supplemented with 10% heat-inactivated FBS (Gibco A3060502), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Gibco 10378016) at 37 °C and 10% CO₂. Doxycycline (Dox; 1 µg/mL) was added to media for at least 20 h to induce expression of the transfected tetracycline-inducible MEG3. Nutlin-3a (Nut; 10 µM) was added to media for at least 24 h to inhibit Mdm2-mediated degradation of p53. For microscopy, 3–5×10⁶ cells were seeded into each well of a chambered 8-well 1.5H coverglass (Ibidi 80827) and allowed to adhere overnight prior to further manipulation. Cells were tested for mycoplasma contamination every three months. U2OS-MEG3 cells were regularly authenticated by qRT-PCR and/or FISH for induction of MEG3 by doxycycline.

**Plasmid construction and transfection**

A modified Tet-On expression system was used to express MEG3, consisting of pBiTetO-MEG3-GFPLoxP and pCMV-rtTA3-IRESpuro. pBiTetO was constructed by replacing the CMV promoter in expression vector pCI with a tetracycline-responsive bi-directional promoter, BtTetO, which was synthesized to contain 7 modified TetO elements flanked by two minimal CMV promoter sequences based on pTet-T2 sequences (GenScript) (44). To facilitate selection of clones, a GFP cDNA with the coding region flanked by two LoxP sites was cloned into pBiTetO to generate pBiTetO-GFPloxP. The MEG3 cDNA in pCI-MEG3 (16) was modified by replacing AATAAA and its downstream poly(A) tail with a genomic DNA fragment containing the MEG3 gene polyadenylation signal. The resultant MEG3 cDNA was then cloned into pBiTetO-GFPloxP to generate pBiTetO-MEG3-GFPloxP. To construct pCMV-rtTA3-IRESpuro, a modified tetracycline responsive transactivator (rTA3) was synthesized with changes in three amino acids including F67S, F86Y, and A209T (16,45) and inserted into
pIRESpuro3 (Clontech Laboratories). Plasmids were verified by sequencing.

For stable transfection, U2OS cells were seeded into 6-well cell culture plates and transfected with pBiTetO-MEG3-GFPloxP and pCMV-rtTA3-IRESpuro at a ratio of 3 to 1 using Mirus TransIT-LT1 according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were re-seeded in P100 dishes with limited dilution. Approximately ten days after treatment with puromycin (2 μg/mL), drug resistant colonies were isolated using cloning rings. Cells from individual clones were treated with or without doxycycline (1 μg/mL) for 24 h. GFP expression was evaluated under a fluorescence microscope. Cells expressing GFP in Dox-treated wells were further examined for MEG3 expression by qRT-PCR. Two sets of primers were used to detect MEG3. The first set detected a fragment near the 5’ end of the MEG3 cDNA: 5’-ATTAGCCCGCCCTTGCTATGC-3’ (forward) and 5’-ATAGGGTATGACAGATCGTC-3’ (reverse); the second set detected the 3’ end of the MEG3: 5’-CTTCAGTGTGTGTAAGGG-3’ (forward) and 5’-TGCTTTGGAAACCGCATACAG-3’ (reverse). The GAPDH gene was used as an internal reference. The primers for detection of GAPDH were: 5’-CATGACATCAAGAGGTGTAAGC-3’ (forward) and 5’-CGTTGTCATATAAAGGGTGATGACAGTCAG-3’ (reverse). Cell clones with suitable MEG3 induction were treated with adenoviruses expressing Cre (Ad-Cre) to remove the floxed GFP. Up to three rounds of virus treatments were needed to completely remove GFP. The removal of GFP was confirmed by qRT-PCR with primer set: 5’-CCACAACGTCTATATCATGGCGG-3’ (forward) and 5’-GGCTCAGGTAAGTGTGTC-3’ (reverse). A total of four clones containing inducible MEG3 were finally obtained and designated as U2OS-MEG3.

Direct stochastic optical reconstruction microscopy (dSTORM)

Fixation

Cells were grown to between 30–90% confluence in chambered coverglasses. Cells were rinsed with prewarmed Dulbecco’s phosphate-buffered saline with calcium and magnesium (DPBS; Corning) twice using near-simultaneous aspiration and injection of liquid to avoid dehydration. Prewarmed fixation buffer (4% (v/v) paraformaldehyde (Electron Microscopy Sciences), 0.1% (v/v) glutaraldehyde (Electron Microscopy Sciences)) was added and incubated in the dark for 15 min. Fixed cells were rinsed with DPBS. Remaining fixative was quenched with 1% (w/v) sodium borohydride (Sigma-Aldrich) for 7 min. (0.1% is typical, but we have observed better suppression of autofluorescence at 1%). Cells were further quenched and washed with 50 mM glycine (Bio-Rad) in DPBS (DPBS-G) 3 times for 10 min each. Fixed cells were stored for up to a week in DPBS at 4 °C.

Immunofluorescence

Cells were permeabilized with 0.2% Triton X-100 (t-octylphenoxypolyethoxethanol, Sigma-Aldrich) in DPBS for 10 min and rinsed with DPBS. Cells were blocked with 5% normal donkey serum (EMD Millipore)/0.02% (v/v) Triton X-100 in DPBS for 4 h at room temperature or overnight at 4 °C. Primary antibodies (rabbit anti-p53 [7F5] (Cell Signaling 2527S, Lot 8), mouse anti-Mdm2 [2A10] (Abcam ab16895, Lot GR324625-5)) were applied at 1:1000 and 1:200 dilutions, respectively, in blocking buffer and incubated overnight at 4 °C. Cells were washed with DPBS 6 times for 5 min each. Secondary antibodies (donkey anti-rabbit IgG (Jackson ImmunoResearch) and donkey anti-mouse IgG (Jackson Immunoresearch)) were labeled as previously described with ATTO 488 (ThermoFisher Scientific) or Alexa Fluor 647 (ThermoFisher Scientific) for a dye ratio of ~1:1 (46). Secondary antibodies were added at 3 μg/mL each in blocking buffer and incubated for 2 h at room temperature in the dark. All subsequent steps were performed in the dark. Cells were washed with DPBS 6 times for 5 min each. Antibody stacks were crosslinked by 4% (v/v) paraformaldehyde in DPBS for 15 min. Remaining fixative was quenched and washed with DPBS-G twice for 5 min each, followed by DPBS twice for 5 min each. Stained cells were stored at 4 °C for up to 2 weeks before imaging.

Combined immunofluorescence and fluorescence in situ hybridization (FISH)
All buffers are RNase-free. Cells were permeabilized with 0.2% Triton X-100 in RNase-free PBS (Corning) for 10 min and rinsed with PBS. No blocking was performed to avoid introducing RNase activity. Primary antibody (rabbit anti-p53, see above) were applied at 1:1000 or 1:200 dilutions, respectively, in PBS and incubated overnight at 4 °C. Cells were washed with PBS 6 times for 5 min each. FISH was performed using buffers and ~20-mer tiled probe sets from Stellaris, according to manufacturer’s protocol. In brief, cells were washed with Wash Buffer A 2 times for 3 min. MEG3-Quasar 670 (Stellaris, custom order) or GAPDH-Quasar 570 (Stellaris SMF-2026-1) probe mixture was mixed 1:1000 in Hybridization Buffer and 100 μL was added per well. Steps from this point forward were conducted in the dark. The chambered coverglass was placed in a pre-warmed humidified chamber (large culture dish with damp paper towels) and incubated at 37 °C for 16 h. Cells were washed 2 times for 15 min each with warm Wash Buffer A in the humidified chamber. Secondary antibodies (donkey anti-rabbit conjugated with ATTO 488, see above) were added at 3 μg/mL each in Wash Buffer A and incubated for 1 h at 37 °C in the humidified chamber. Cells were washed 2 times for 2 min each with Wash Buffer B, then 2 times for 5 min each with PBS.

Imaging

Imaging buffer containing 10 mM cysteamine (2-mercaptoethylamine, MEA; Sigma-Aldrich), 3 U/mL pyranose oxidase from Coriolus sp. (Sigma P4234), and 90 U/mL catalase was freshly prepared in STORM buffer. Cysteamine stock solution was previously titrated to pH 8 and aliquots frozen. Precipitate in pyranose oxidase/catalase 100x enzyme stock solution was cleared by centrifugation at over 14,000×g prior to use. STORM buffer was composed of 10% (w/v) glucose, 10 mM sodium chloride, and 50 mM Tris hydrochloride (pH 8.0). We found the pyranose oxidase buffer (first described in (47)) to be superior to the standard glucose oxidase buffer. This buffer allowed longer imaging times due to minimal pH change, and the enzyme stock lasted several months at 4 °C with no observable decline in imaging quality. 10 mM cysteamine was selected for superior imaging characteristics with different dyes (48). PBS was replaced with the imaging buffer and the slide was mounted on the stage with type F immersion oil (refractive index = 1.515) on a Nikon Ti2 Eclipse inverted microscope. The microscope was equipped with a 100x 1.49 NA APO-TIRF objective with automatic correction collar and a Nikon NSTORM system including 405 nm (20 mW), 488 nm (70 mW), 561 nm (70 mW), and 647 nm (125 mW) lasers, a quadband excitation-emission filter, and a Hamamatsu ORCA Flash4.0 V2 S-CMOS camera. Nikon Elements 5.02 was used for image acquisition. A 10x10 tiled (with 15% overlap) widefield fluorescence image (~790x790 μm²) was obtained with 1 s exposure using GFPHQ, TexasRedHYQ, or Cy5HYQ filter cubes, from which random individual cells were selected for dSTORM imaging. At least 11000, 256x256 pixel (160 μm/pixel) frames were collected with 10 ms exposure time at 100% laser power with lasers in highly inclined and laminated optical sheet (HILO) configuration (49). Each channel was collected sequentially from longest wavelength to shortest.

Data analysis

Localizations were identified from STORM image stacks using Nikon Elements 5.02 (NSTORM 4.0), with a peak height threshold of 250. Localization lists were exported as tab-delimited text files.

Localization data was processed with custom code written in the freely available Julia scientific computing language (v1.4) (50). Localizations identified in the first 100 frames, while fluorophores are being placed into the “off” state, were excluded. Localizations identified in the last 10 frames were also excluded due to artifacts caused by the change in optical configuration. For each image, a grouping algorithm (SI Algorithm 1; Figure S5) was applied to each channel to combine repeated blinking from single fluorophores and localizations that may be associated (e.g., another fluorophore on same secondary antibody, another secondary antibody on the same primary antibody, another primary antibody on a multimer). The first stage of the grouping algorithm iteratively identified local density maxima by searching a 34.2 nm radius and within a temporal window of 500 frames (5.0 s) of each localization for the localizations with the most neighbors, combining those localizations within the radius of the maxima,
and repeating until all localizations were assigned to a group. The 34.2 nm radius limit was derived from a simulation of the possible orientation and positions of fluorophores in an antibody stack, to account for possible motion of the antibody stack and multiple fluorophores on the stack. The temporal window was applied to account for longer-scale on/off cycles of the fluorophores, as first described for PALM data (43), and was chosen semi-empirically by testing a range of values and selecting the smallest value that merged the most localizations (i.e., where the slope starts to decrease before the plateau) (Figure 10A) and where the merge results appeared suitable (e.g., few temporally separated clusters of localizations were merged).

In the second stage, grouped localizations were merged if they were found within 200 nm of each other by a similar local density maxima search algorithm to further reduce redundancy from autocorrelated localizations. The products of this grouping algorithm were termed “molecules.” The position of the resulting molecule was the mean of its component localizations’ positions, and its linear localization accuracy was the mean of the accuracy for its component localizations divided by the square root of the number of component localizations.

The molecules were paired between channels by an exclusive cross–nearest neighbor algorithm (i.e., closest pair found and then removed, next closest pair found and then removed…; SI Algorithm 3) to obtain a distance distribution between the two sets of molecules. Two analytical approaches were applied, simple and sophisticated. First, the median paired distance was calculated for each cell. Second, a novel approach was developed to control for local density, based on a similar approach applied to single-molecule conventional fluorescence microscopy (29). Random permutations (10,000) of the molecules in the local (800 nm radius) neighborhood around each potential binding pair were generated and the closest pairwise distance in each permutation was calculated to create a Monte Carlo estimation of the distribution of distances due to local density (SI Algorithm 4). The local neighborhood radius of 800 nm was semi-empirically chosen based on testing multiple window sizes with the algorithm and choosing the value that provided a balance of sensitivity (smaller value) and robustness (less inter-sample change as parameter changes) (Figure 10B). The fraction of permutations with a closest distance less than the observed distance was the percentile rank score, indicating the probability of chance association given the local density of both molecule types. Finally, the fraction of pairs within the maximum binding distance (200 nm) and with a probability of chance association of less than 0.1 was calculated for each cell. The maximum binding distance was chosen based on knowledge of the size of the target.
molecules (up to 20 nm across) and the size of the antibody stacks (up to 70 nm), with allowance for error. Binding fraction for each cell was normalized using a positive and negative control generated from each cell. For the positive control (simulated 100% binding), each putative pair had one member randomly moved to within 80 nm of its partner. For the negative control (simulated 0% binding), all molecules were randomized within 800 nm of their original position, to maintain large-scale structure. For the negative control, between 5 and 30 iterations were conducted, with more iterations when there were fewer molecule pairs to dampen variance, and the median fraction bound of these trials was taken as the zero point.

These data were tested for significance by two-way fixed-factor ANOVA with 2 nested factors (cells within replicates within conditions), with \( \alpha = 0.05 \), using the SimpleANOVA.jl (v0.6.0) Julia package created by the authors. Data was checked for extreme outliers, heteroscedasticity, and normality of residuals, and were determined to be reasonable. Plots were generated with StatsPlots.jl (v0.14.0) and assembled with Adobe Illustrator (24.0).

**Code availability**

All the code generated specifically for this manuscript is written in the Julia language and available in the repository at doi: 10.5281/zenodo.3893264. Supporting packages can be obtained within Julia from its public package registry.

**Data availability**

Raw STORM data files are stored on a local server. STORM localization list text files are available in the repository at doi: 10.5281/zenodo.3892995.
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