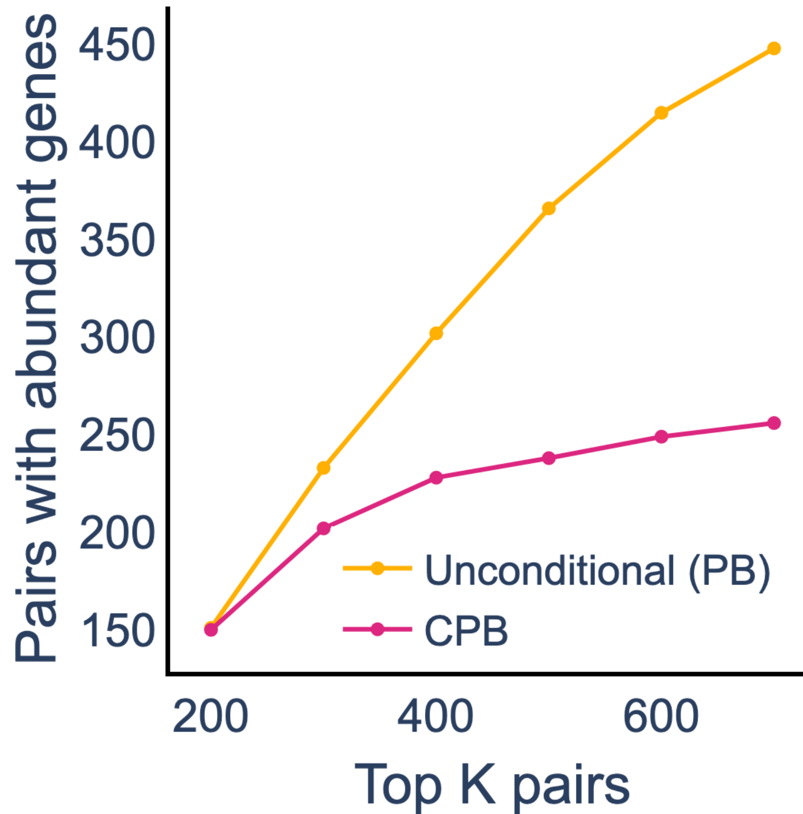
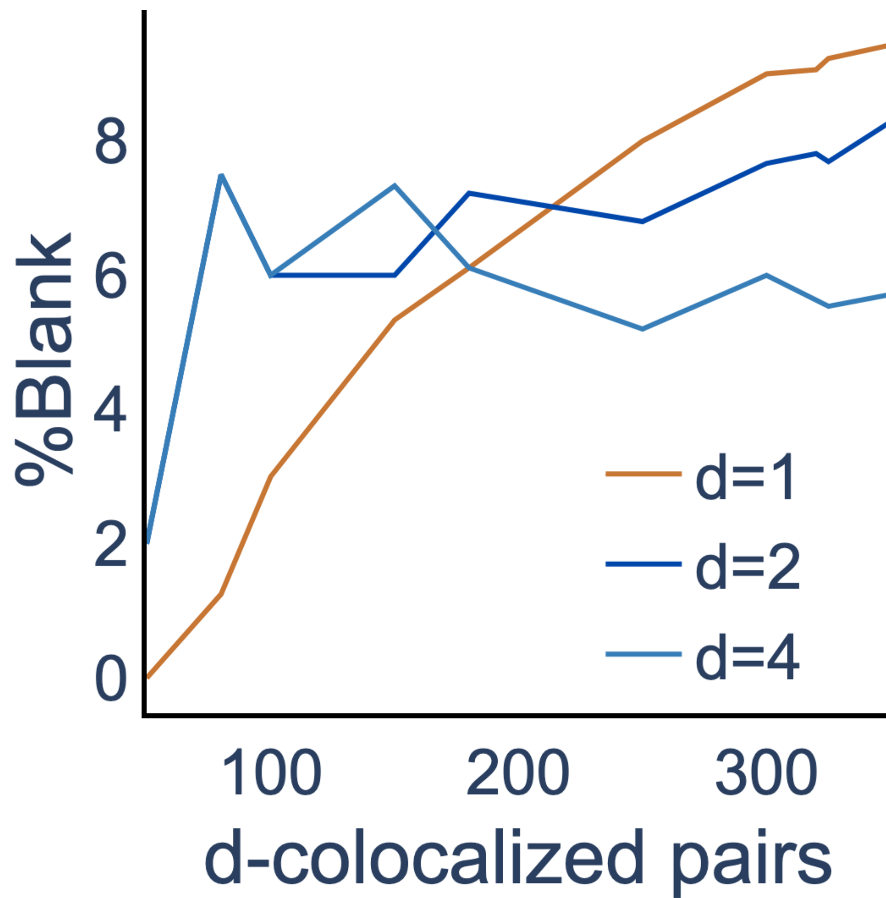
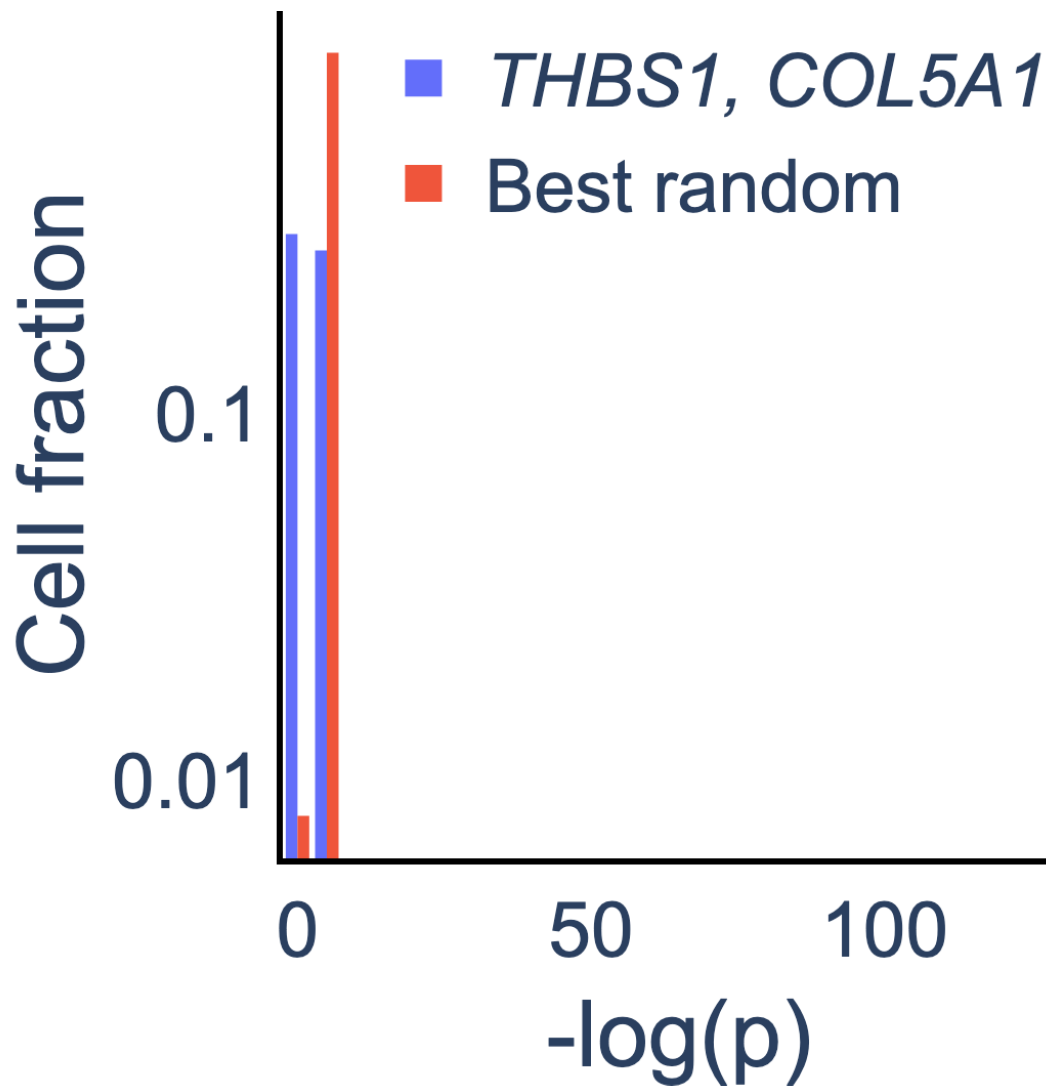
Supplementary Figures



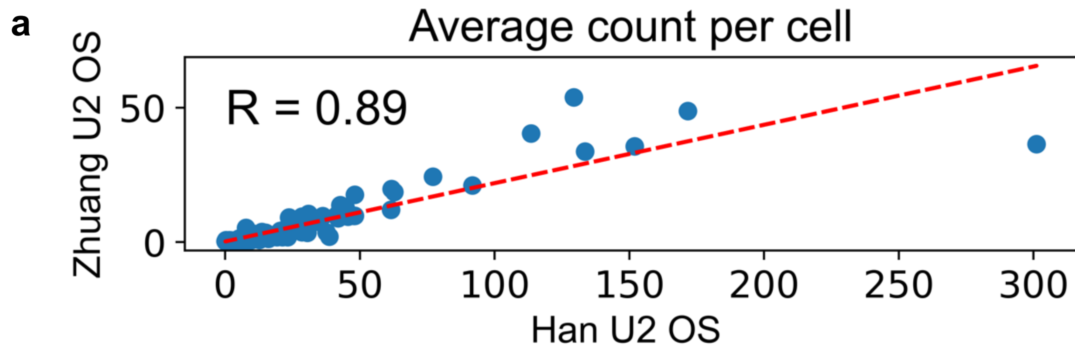
Supplementary Figure 1. Comparison of Conditional Poisson Binomial (CPB) test and Poisson Binomial (PB) test. The y-axis shows how many of the top K gene pairs identified by either test include an "abundant" gene, defined as one of the five highest expressed genes across cells. (Results are for U2OS cell line data.) We note that the gene pairs identified as significant by the PB test are dominated by abundant genes, and the CPB test mitigates this bias.

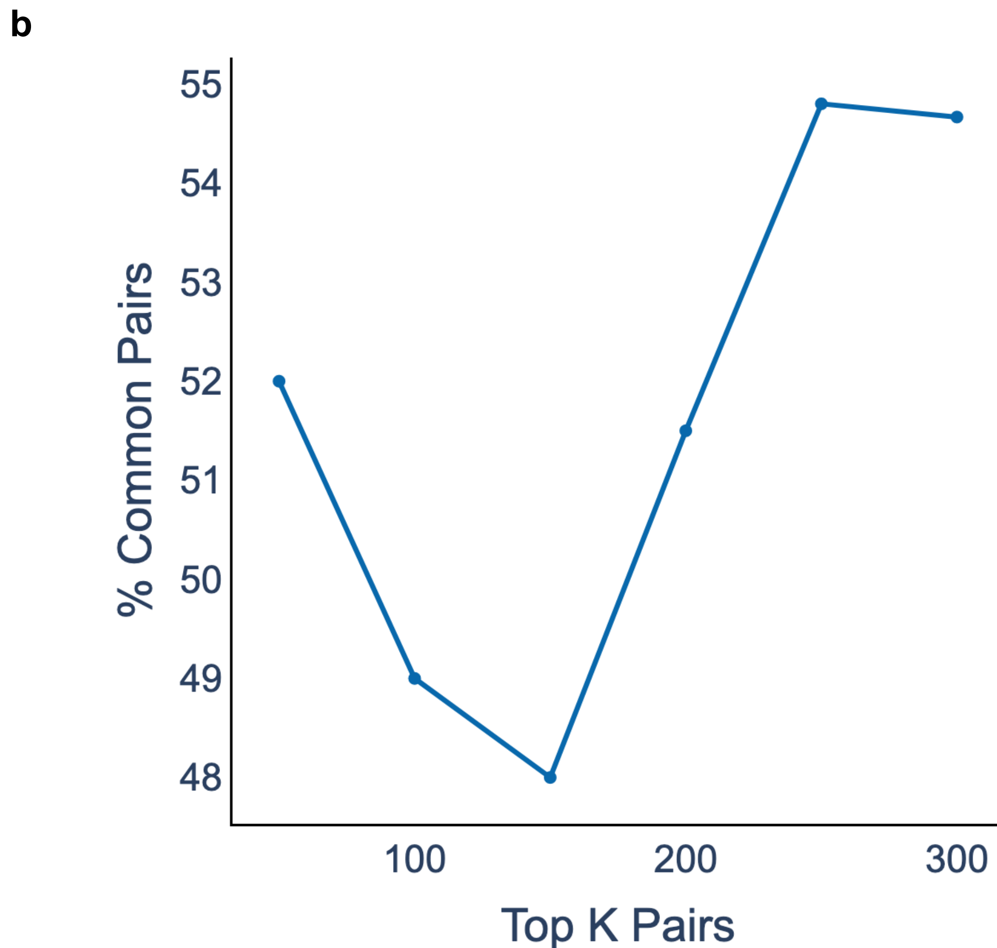


Supplementary Figure 2. Y-axis shows the percentage of d-colocalized gene pairs (reported by CPB test) that include at least one blank "gene", at varying p-value thresholds. Such pairs are presumed to be false positive predictions. InSTAnT predicts ~300 d-colocalized pairs at d = 4 μm with low blank fraction (<10%).

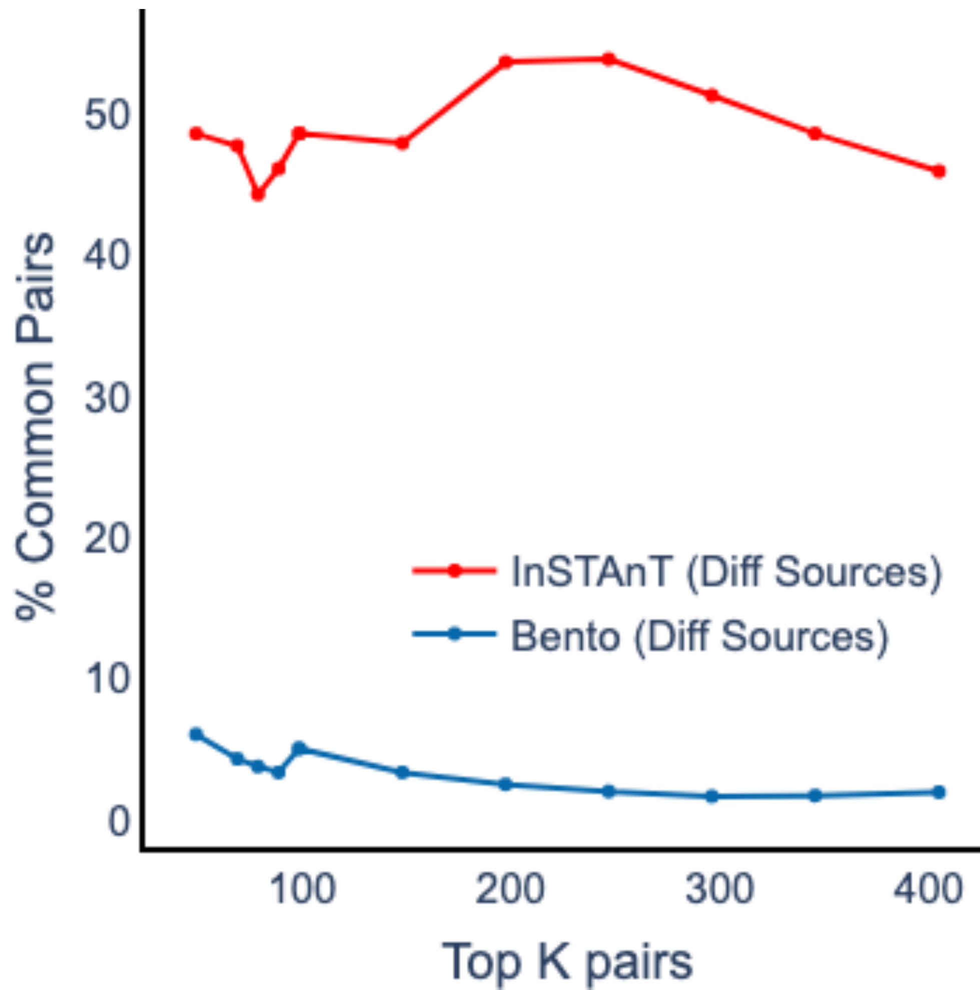


Supplementary Figure 3. Blue: Histogram of -log(p-value) obtained from PP Test for the gene pair *THBS1, COL5A1* over 3237 cells. Also shown (red) is the histogram of -log(p-value) for the "best" gene pair identified from randomized data where gene identities were shuffled among all transcripts of that cell. The best gene pair is defined as the pair with lowest PP p-value in randomized cell. The comparison illustrates how the CPB test detects the persistent appearance of a proximal pair across many cells.

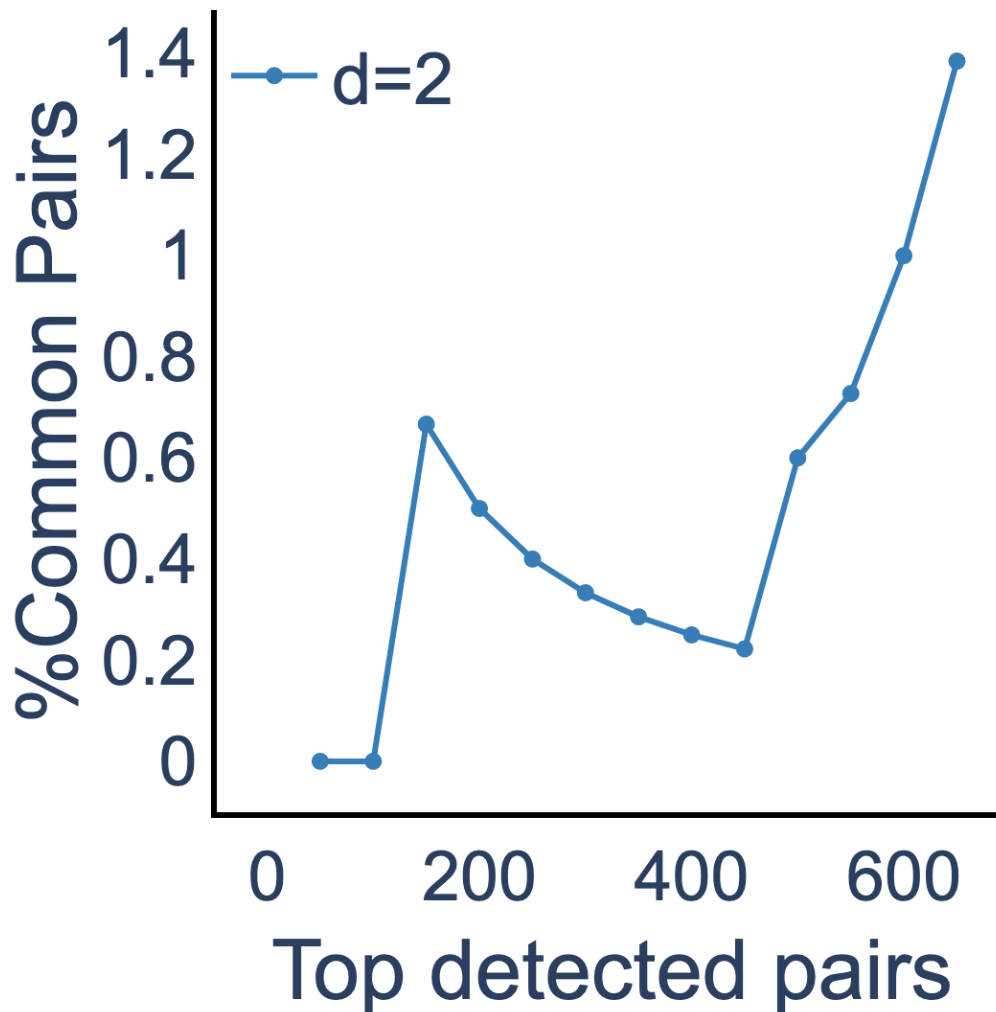




Supplementary Figure 4. Reproducibility of data and findings between sources (labs). (a) shows high reproducibility across MERFISH data on U2OS cell lines from the Zhuang lab ("Zhuang U2 OS") and our data ("Han U2 OS"), in terms of the average transcript count per cell for each gene. Each dot corresponds to a gene. (b) shows that ~50% of top correlated gene pairs overlap across Zhuang and Han U2OS data. Pearson correlation was performed on each gene's expression data.

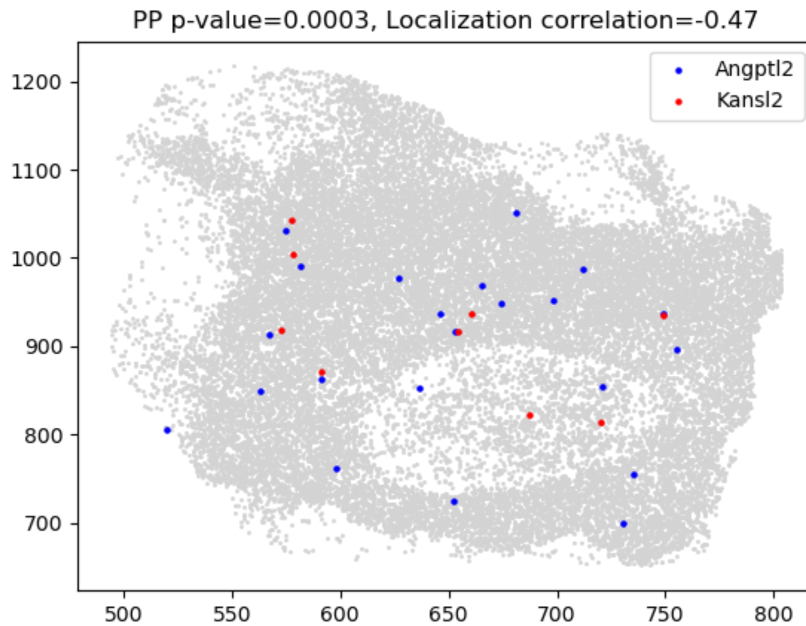


Supplementary Figure 5. Reproducibility of top gene pairs across datasets (Zhuang U2OS and Han U2OS) for InSTAnT (CPB test) and Bento. Y-axis shows the percentage of top K gene pairs reported on either data set that are common between the data sets. Results labeled as “Bento” correspond to an aggregation of Bento results across cells, whereby a gene pair’s colocation quotient scores across all cells are averaged and pairs are ranked by this average.

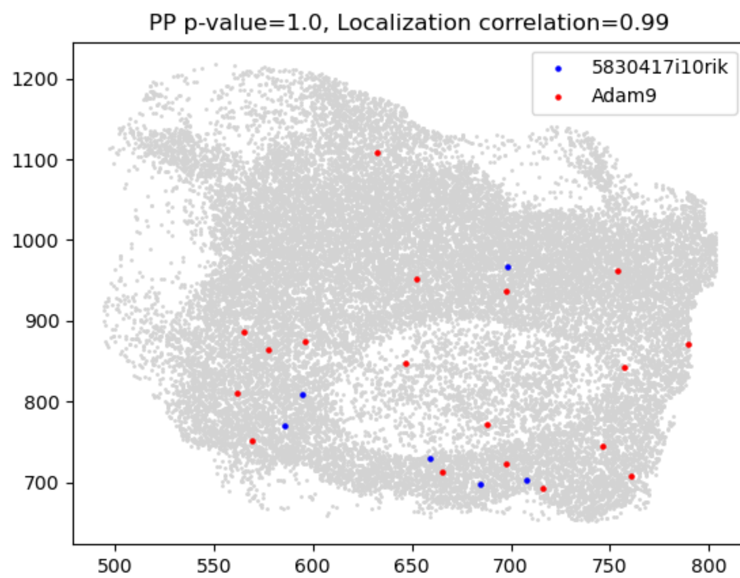


Supplementary Figure 6. Comparison of gene pairs detected by InSTAnT (CPB test) and an alternative method based on Localization features, on Seqfish+ data from NIH/3T3 cell line. The y-axis shows the percentage of top K pairs (x-axis) detected by the two methods that are common to both reported lists. The "Localization feature"-based method uses 13 localization features computed by Bento (methods) for each gene in a cell. Non-negative factorization decomposition of the tensor of localization features (genes x features x cells) was performed to obtain latent representation of each gene which was then used to obtain gene-gene similarities of localization in aggregate across all cells.

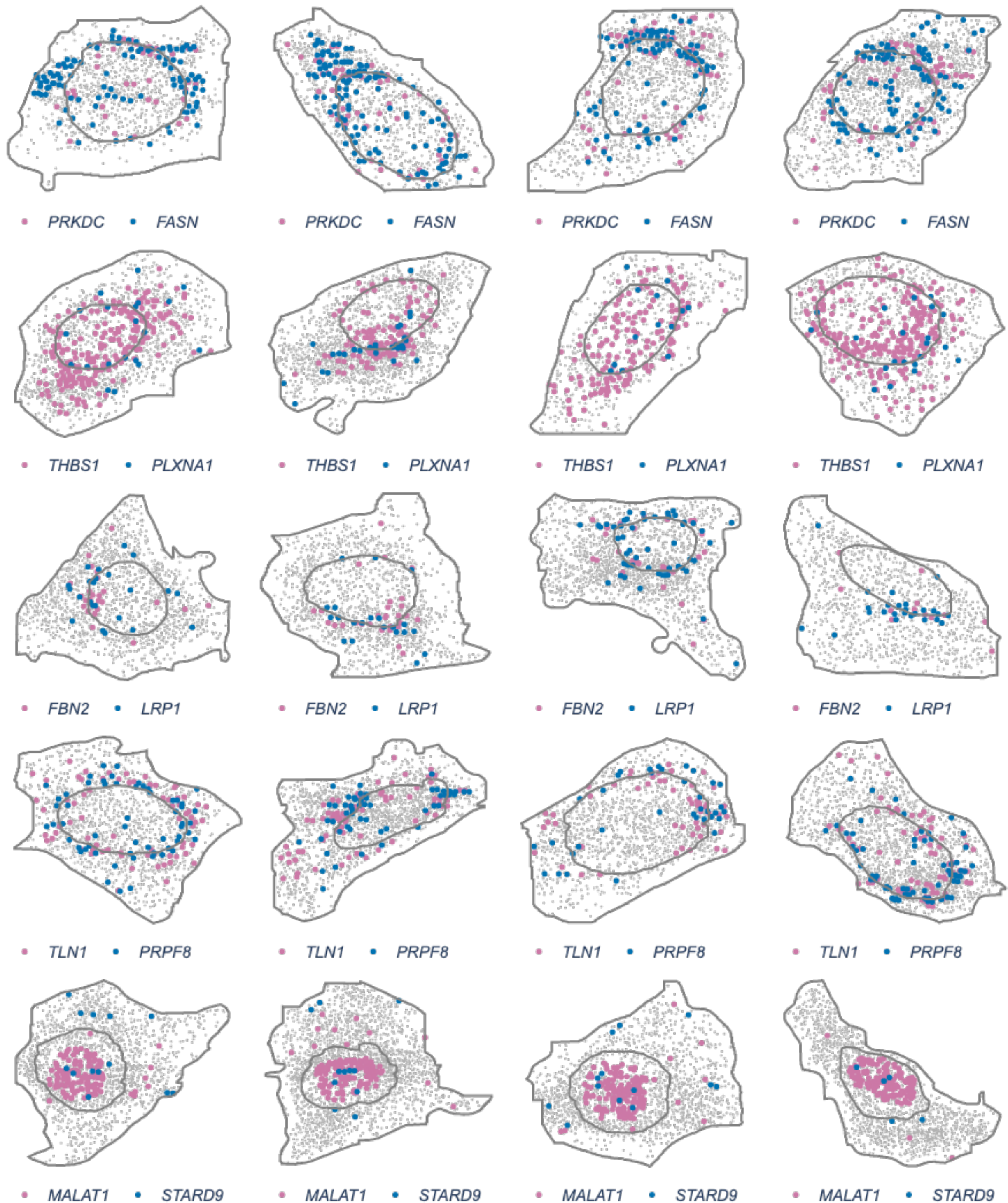
a



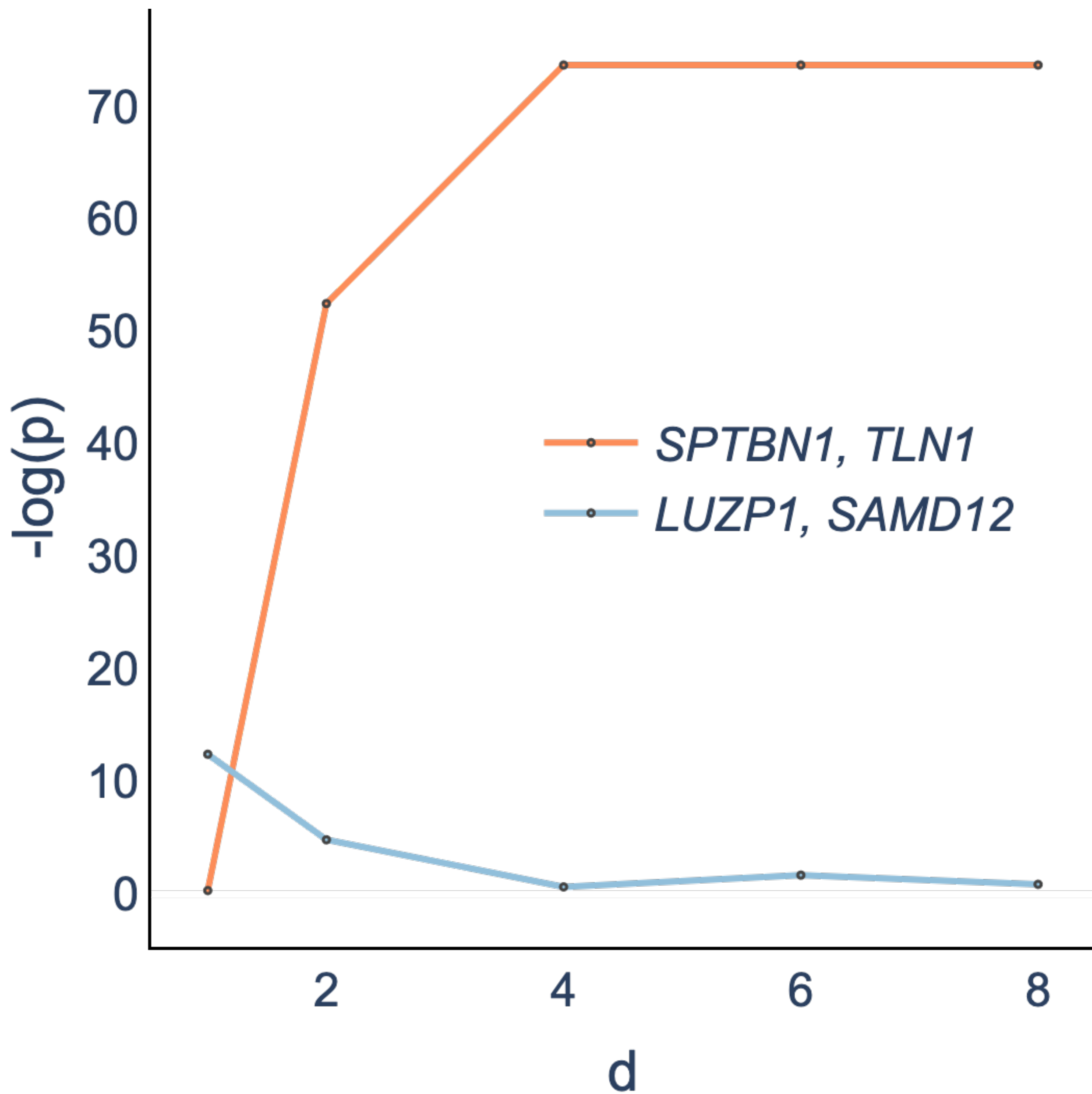
b



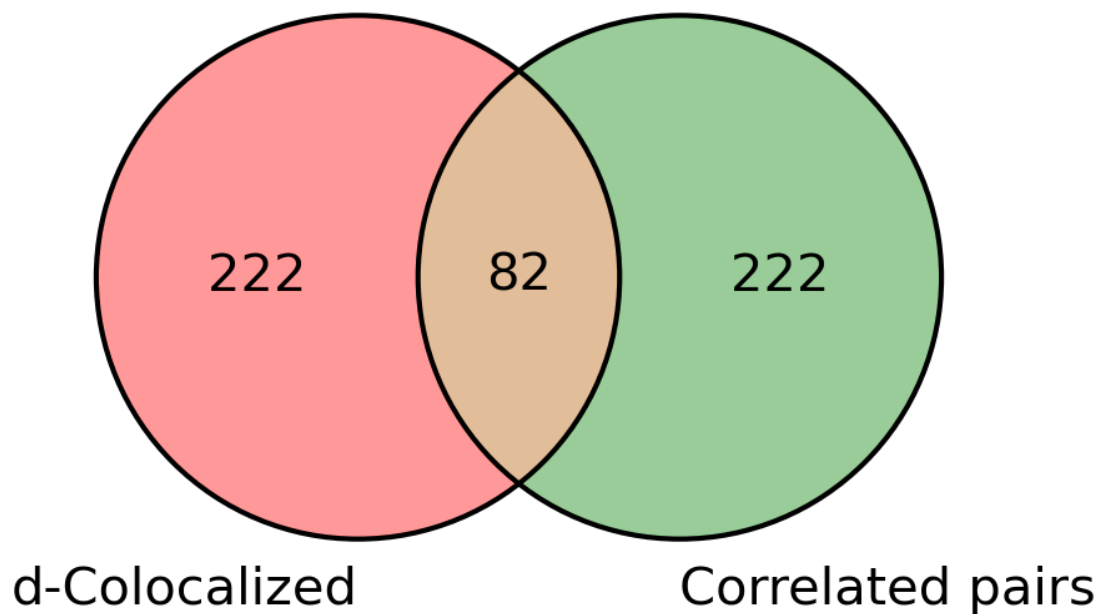
Supplementary Figure 7. Examples of differences between findings of the PP test and an alternative approached based on localization features. (a) An example where a gene pair is reported as a proximal pair in a cell, according to the PP test (*d*=2), while the correlation coefficient between localization features of the two genes in this cell is low. (b) An example where a gene pair is not reported as a proximal pair in a cell by the PP test (*d*=2), but the two genes have similar localization features.



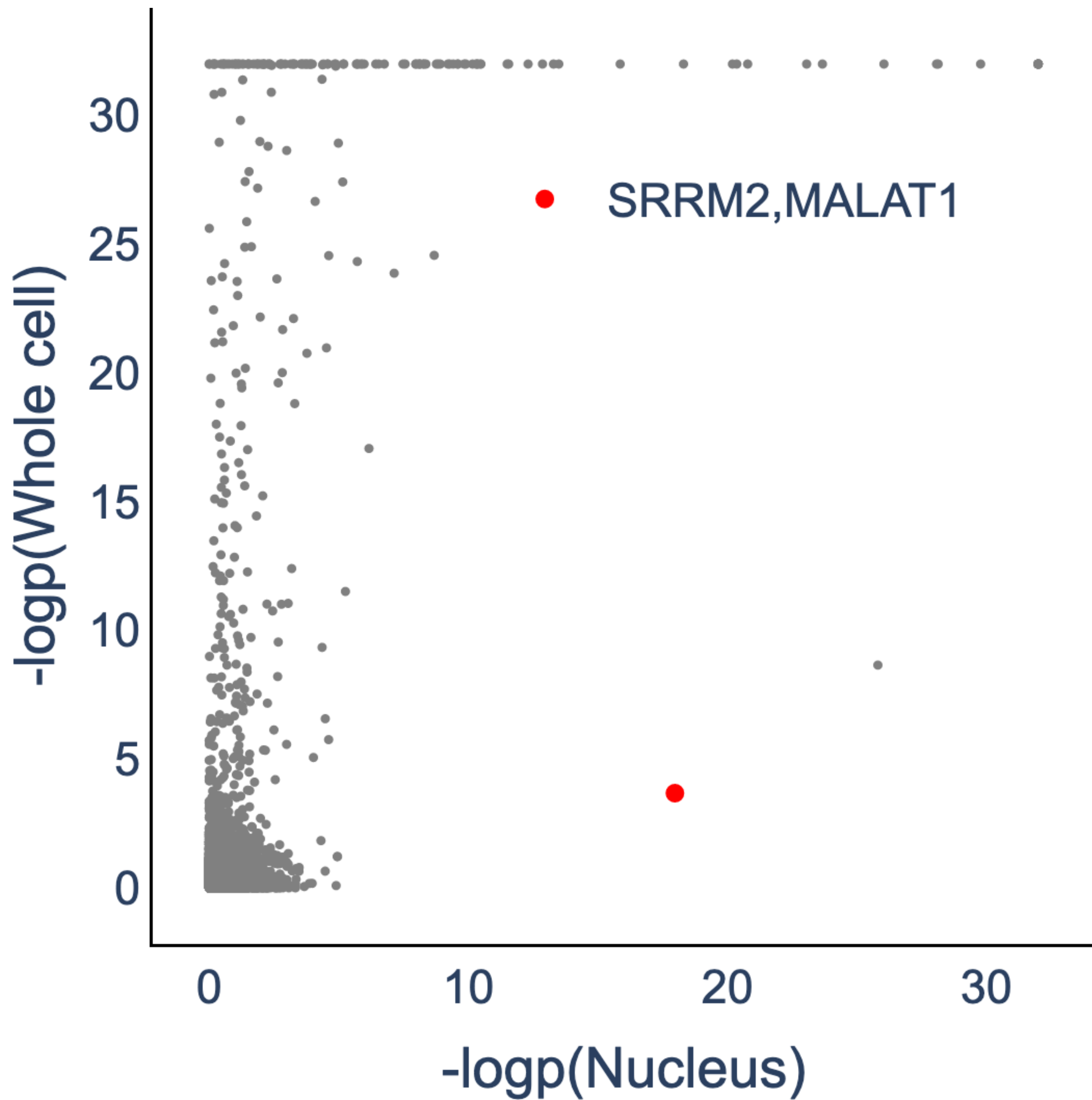
Supplementary Figure 8. Visualization of selected d-colocalized pairs (obtained using CPB test) in sample cells. Results are for MERFISH data on U2OS cells. In each sample cell, a proximal pair (gene pair with significant PP test p-value in that cell) is shown, with transcripts of the two genes of the pair in different colors. The nuclear boundary is also shown.



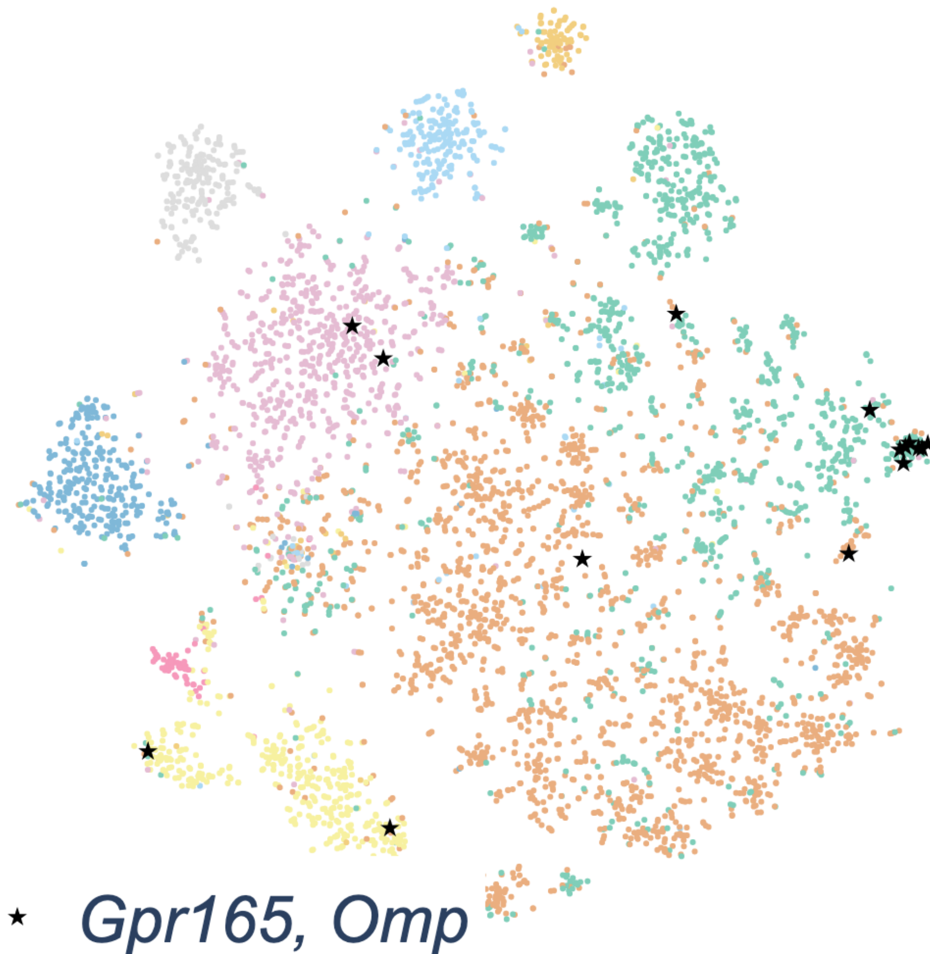
Supplementary Figure 9. Examples of scale-dependent d-colocalization detected using CPB test. Each line shows how -log(p-value) of the CPB test for the selected gene pair varies as *d* varies between 1 and 6 microns. The pair SPTBN1-TLN1 is detected most strongly at d $\geq$ 4 microns while colocalization of LUZP1-SAMD12 is most significant at d=1 micron.



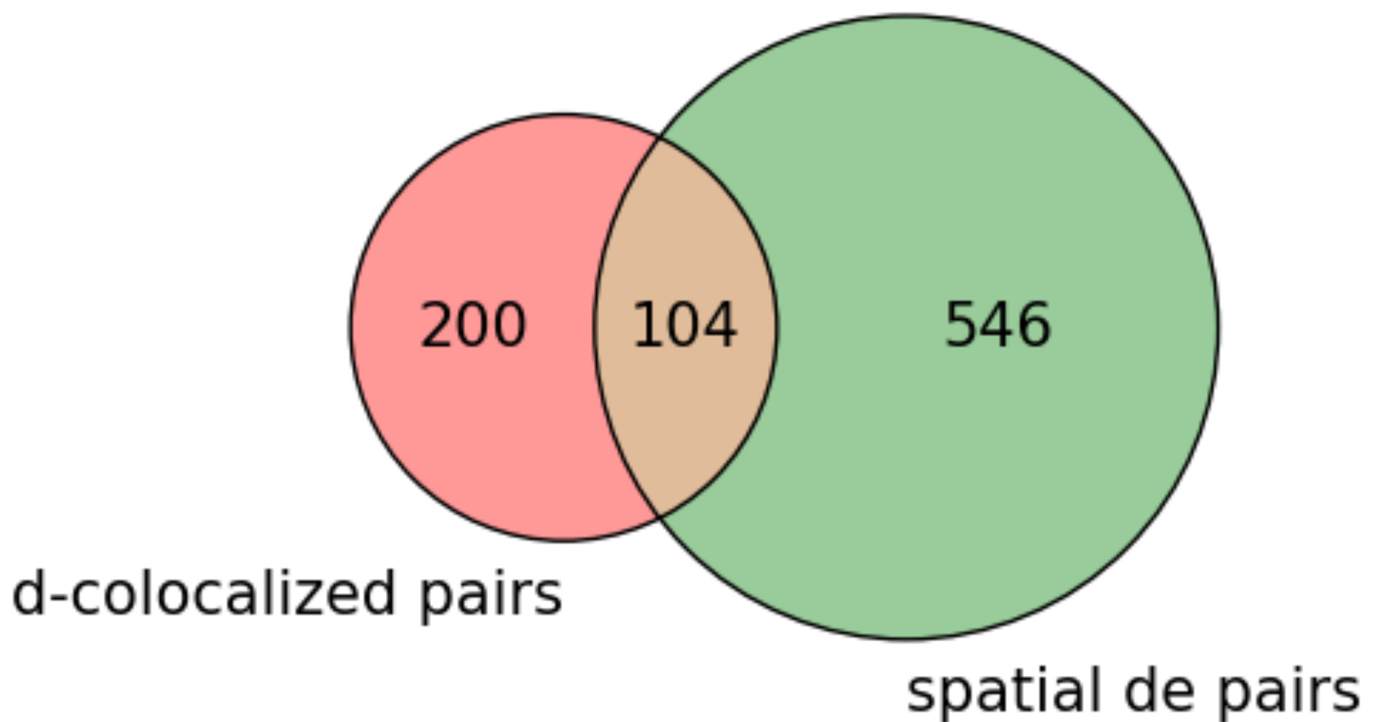
Supplementary Figure 10. Overlap of the set of d-colocalized gene pairs (InSTAnT CPB test on U2OS data) with the top 304 co-expressed gene pairs. Co-expressed gene pairs are identified based on Pearson correlation of whole-cell transcript counts, and the top 304 pairs are taken so as to match the size of the d-colocalization map.



Supplementary Figure 11. Negative logarithms of p-values of CPB test on all gene pairs, in the whole-cell mode (y-axis) and in the intra-nucleus mode. P-values are more conservative in the intra-nucleus mode, in part because fewer transcripts are analyzed (only those within nuclei) and in part because this mode de-emphasizes the colocalization of nucleus-enriched genes. The largest adjustment of significant is seen for the pair *SRRM2-MALAT1* (red dot), which is not d-colocalized at the whole cell level but is d-colocalized in intra-nucleus mode.



Supplementary Figure 12. Example of a "category 2" (see text) gene pair whose colocalization is specific to excitatory neurons. Shown is a tSNE plot of cells in the mouse brain data set, with different cell types in different colors, and excitatory neurons in green Each black star is a cell in which the gene pair Gpr165-Omp is a significant proximal pair according to the PP test.



Supplementary Figure 13. Overlap between *d*-colocalized pairs and “SpatialDE pairs” on U2OS MERFISH data. A SpatialDE pair is defined as any gene pair that contains at least one of the top 5 spatially varying genes obtained using SpatialDE.

Supplementary note A: SeqFISH data analysis

We used InSTAnT to analyze SeqFISH+ data on NIH/3T3 cell line (of mouse fibroblast origin) and constructed a global d-colocalization map (d = 2 micron) using the CPB test. The data set included 3726 genes; thus 6943401 gene pairs were tested for d-colocalization using the CPB test.

Of the 15 most significant gene pairs (**Table A**), 11 pairs involved genes that are related to extracellular matrix and/or cell adhesion/migration, which is consistent with the key role fibroblasts play in ECM organization and thus to cell adhesion and proliferation. The most prominent genes among the 11 pairs are Fn1 (fibronectin), a key component of ECM involved in cell adhesion, and Col1a1 (alpha-1 chain of type I collagen), the most abundant collagen in the ECM.

Table A (below). Top 15 gene pairs by CPB test p-value.

g1g2	p_value (CPB)	# cells with proximal pairs	#cells with both genes present	fraction of cells with proximal pairs
5830417i10rik, Gon4l	3.18E-153	71	85	0.835294118
Polr2a, Prpf8	7.77E-113	62	163	0.380368098
Col1a1, Fn1	1.42E-103	77	179	0.430167598
Fbln2, Fn1	2.62E-51	45	177	0.254237288
Col1a1, Fbln2	8.85E-49	43	177	0.242937853
Mki67, Ranbp2	7.32E-41	22	103	0.213592233
Skiv2l, Ttc37	1.04E-37	20	56	0.357142857
Col1a1, Col6a2	5.25E-35	32	179	0.17877095
Ddr2, Sh3pxd2a	1.17E-34	17	166	0.102409639
Bgn, Col1a1	1.17E-30	32	178	0.179775281
Col6a2, Fn1	3.91E-30	29	179	0.162011173
Cyb5r3, Sh3pxd2a	8.73E-30	17	175	0.097142857
Fn1, Vcl	1.63E-27	23	173	0.132947977
Sh3pxd2a, Trak2	8.70E-24	14	136	0.102941176
Bgn, Col6a2	9.82E-24	24	178	0.134831461

Examining a larger set of 109 gene pairs that received a CPB test p-value of < 1E-10 (Bonferroni corrected p-value 0.001, FPR < 1%) (Supplementary Table 5). We noted a large module of genes related to extracellular matrix (**Figure 3n**), encoding proteins that are either components of the ECM or known for remodeling ECM or mediating ECM-cell interactions (see **List A** for details).

There is some evidence that mRNA of ECM proteins such as Fn1 and collagens are localized to perinuclear regions[4,5], the rough endoplasmic reticulum and Golgi apparatus, where localized protein synthesis, post-translational modifications and/or secretion are known to happen[6-9]. Similarly, activation of receptors for ECM components may lead to localized translation[10,11], which may underlie the observed RNA localization patterns. Our findings of several gene pairs of ECM-related proteins being colocalized suggests that similar phenomena may be more widespread than currently recorded.

List A: Literature evidence of involvement in extracellular matrix structure and function, for a selection of genes (shown as green nodes in Figure Y1). Papers with such evidence are cited, and text exerpts from those papers are reproduced verbatim or paraphrased below.

Aebp1: AEBP1 encodes the aortic carboxypeptidase-like protein (ACLP) that associates with collagens in the extracellular matrix (ECM) and has several roles in development, tissue repair, and fibrosis. ACLP is expressed in bone, the vasculature, and dermal tissues and is involved in fibroblast proliferation and mesenchymal stem cell differentiation into collagen-producing cells[12].

Axl: AXL modulates extracellular matrix protein expression and is essential for invasion and metastasis in endometrial cancer[13]. AXL contains an extracellular domain composed of two immunoglobulin-like domains and two fibronectin type III domains[14,15]. The extracellular domain resembles that of adhesion molecules, and overexpression of AXL causes an adhesive phenotype.

BGN (Biglycan) is a small proteoglycan that is primarily associated with the extracellular matrix (ECM)[16,17]. BGN interacts with other ECM components, such as collagen, to regulate ECM organization and structure.

Calu: Extracellular calumenin inhibits cell migration[18].

CTGF: Connective tissue growth factor (CTGF/CCN2) is a cysteine-rich, extracellular matrix (ECM) protein[19].

CYR61: CCN1 (CYR61) is a dynamically expressed, multifunctional matricellular protein. The term “matricellular protein” was first introduced by Bornstein in 1995 to describe a group of extracellular matrix (ECM) proteins that play minimal roles in matrix structural integrity, but regulate a multitude of cellular responses[20].

DDR2: Discoidin domain receptor 2 (DDR2) is a collagen receptor belonging to receptor tyrosine kinase (RTK) family. It is a powerful regulator of collagen deposition in the extracellular matrix (ECM). The oligomerization of DDR extracellular domain (ECD) proteins can affect matrix remodeling by inhibiting fibrillogenesis[21].

EMP1: Extracellular Matrix Protein 1 gene.

FBLN2: Fibulin-2 (FBLN2) is a secreted extracellular matrix glycoprotein which has been associated with tissue development and remodeling[22].

FBN1: Fibrillin-1 is a calcium binding protein of extracellular matrix[23].

FLNA: Filamin A regulates the organization and remodeling of the pericellular collagen matrix[24]. It has been hypothesized that FLNa-β1 integrin complexes could serve as a mechanical or biochemical link that couples the actin cytoskeleton to the ECM and regulate cell morphogenesis in response to the stiffness of the extracellular matrix[25].

FLNB: Filamin B knockdown enhances ECM degradation[26].

FN1: Fibronectin (FBN) is an extracellular matrix (ECM) component that, through binding integrin receptors of the cell surface, acts as a key player of the communication between the intra and the extracellular environment, thus controlling cell behavior[27].

HSPG2: The extracellular matrix proteoglycan (ECM) perlecan, also known as heparan sulfate proteoglycan 2 or HSPG2, is one of the largest (> 200 nm) and oldest (> 550 M years) extracellular matrix molecules[28].

ITGB1: ITGB1 functions facilitating cell–cell and cell–extracellular matrix interactions[29].

LOX: Lysyl oxidase (LOX) is a copper-dependent amine oxidase whose primary function is the covalent crosslinking of collagens in the extracellular matrix (ECM)[30].

LOXL3: LOXL3 is a member of the lysyl oxidase (LOX) family that are copper-dependent amine oxidases, generating covalent cross-links to stabilize polymeric elastin and collagen fibers in the ECM[31].

LTBP2: Latent TGF-β-binding protein-2 (LTBP-2) is an extracellular matrix protein associated with microfibrils[32].

NID1: NID1 acts as a linker between laminins, collagens and proteoglycans in the extracellular matrix and binds to cell surface integrins, involving in establishing and maintaining the basement membrane and tissue architecture. The IHC staining showed that NID1 mainly expressed in extracellular matrix[33].

P4HB: P4HB (Prolyl 4-hydroxylase subunit beta) is a subunit of P4H. Prolyl 4-hydroxylase and its role in collagen synthesis[34]. P4HB binds Collagen chains. (Source: reactome.org)

PDIA3: PDIA3/ERp57 promotes a matrix-rich secretome that stimulates fibroblast adhesion through CCN2. Loss of PDIA3 function, either by pharmacological inhibition in HDF or in *Pdia3*$^{-/-}$ mouse embryo fibroblasts (*Pdia3*$^{-/-}$ MEFs), led to alterations in the composition of cell-derived extracellular matrix[35].

POSTN: POSTN (Periostin) is an ECM (extracellular matrix) protein involved in tissue remodeling in response to injury and a contributing factor in tumorigenesis[36].

PTX3: This suggests that the octameric structure of PTX3 acts as nodal molecule in cross-linking hyaluronic acid in the extracellular matrix[37].

REST: Rest ablation impairs the extracellular matrix (ECM) component[38].

SERPINE1: Human PAI-1 is the product of the *SERPINE1* gene. PAI-1 activities, including fine regulation of pericellular proteolysis, ECM remodeling, and cell motility[39].

SERPINF1: Also known as PEDF. PEDF has been reported to bind to extracellular matrix (ECM) components such as collagens and glycosaminoglycans (GAGs)[40].

SERPINH1: CyPA interacts with SERPINH1 to promote extracellular matrix production. Silencing SERPINH1 expression reversed the upregulation of ECM proteins[41]. Hsp47, also called "SERPINH1," is an ER-resident chaperone that is essential for the proper assembly of the triple-helical procollagen molecules, which eventually are transported across the Golgi apparatus to the extracellular space[42].

Tks5/Fish appears to be required for podosome formation, for degradation of the extracellular matrix. Podosomes are thought to promote the invasive properties of cells, by clustering proteases involved in extracellular matrix degradation. The authors show that previously shown that the scaffolding protein and Src substrate Tks5/Fish localizes to podosomes[43].

THBS1: Thrombospondin 1 (TSP1) is a matricellular extracellular matrix protein that has diverse roles in regulating cellular processes important for the pathogenesis of fibrotic diseases. Matricellular proteins are components of the extracellular matrix (ECM) that interact with cells and other ECM components[44].

TNC: The Extracellular Matrix Glycoprotein Tenascin C and Adult Neurogenesis. Tenascin C (TnC) is a glycoprotein highly expressed in the extracellular matrix (ECM) during development and in the adult central nervous system (CNS) in regions of active neurogenesis[45].

TRAK2: Messenger RNA (mRNA) compartmentalization within the cytosol is well-recognized as a key mechanism of local translation-mediated regulation of protein levels, but whether such localization could be a means of exercising non-coding mRNA function is unknown. Here, we explore non-coding functions for mRNAs associated with focal adhesions (FAs), cellular structures responsible for mediating cell adhesion and response to changes in the extracellular matrix (ECM). Using high-throughput single molecule imaging and genomic profiling approaches, we find that mRNAs with distinct sequence characteristics localize to FAs in different human cell types. Notably, ~85% of FA-mRNAs are not translationally active at steady state or under conditions of FA dissolution or activation. Untranslated mRNA sequences are anchored to FA based on their functional states by the RNA binding protein, G3BP1, forming biomolecular granules. We have therefore uncovered a novel, non- coding role for mRNAs as scaffolds to maintain FA structure and function, broadening our understating of noncanonical mRNA functions. We found that TRAK2, an untranslated FA-mRNA, did

not form its polypeptide at new FA sites nor in other cellular compartments when compared to untreated cells[46].

VCAN: The extracellular matrix (ECM) proteoglycan, versican[47]. In adults, this proteoglycan serves as a structural macromolecule of the extracellular matrix[48].

VCL: Vinculin is filamentous (F)-actin-binding protein enriched in integrin-based adhesions to the extracellular matrix (ECM)[49].

List Y3: Literature evidence for possible relationship to extracellular matrix for a selection of genes (not colored in green in Figure Y1).

Bard1: BARD1 repression by siRNAs, mitigates the interference of cytotrophoblasts with cell adhesion of collagen matrix-dependent epithelial cells, suggesting a role of BARD1 isoforms in extracellular matrix remodelling and in cytotrophoblasts invasion[50].

KIF1C: The kinesin KIF1C is known to regulate podosomes, actin-rich adhesion structures that remodel the extracellular matrix. KIF1C translocation to the cell periphery intensifies and KIF1C accumulates both in the proximity of peripheral microtubules. Focal adhesions are complex assemblies containing more than 150 different proteins that physically link the end of actin stress fibers to extracellular matrix proteins. The microtubule-dependent regulation of podosomes, specialized adhesion structures in human macrophages, requires Kif1C, a kinesin-3[51].

MKI67: The MKI67 gene encodes a protein called Ki-67, which is a widely used marker for cell proliferation. Fibroblasts are responsible for synthesizing and maintaining the extracellular matrix in tissues.

PLEC: Plectin (PLEC) is an intracellular structural protein which act as anchoring points for ECM proteins[52].

PRPF8: *PRPF8* silencing also induced reorganization of the cytoskeleton by drastically reducing stress fiber formation[53] .

Supplementary Note B: Behavior analysis

We used the Differential Colocalization routine of Instant to identify gene pairs that were d-colocalized specifically in hypothalamus preoptic region of brains of male mice exhibiting aggressive behavior (compared to naïve mice). This routine not only tested if the cells exhibiting the d-colocalization are enriched in one group (aggression) versus another (naïve), it also used multiple statistical procedures to ensure that this is not a side-effect of one of the genes exhibiting group-specific expression. Three gene pairs met these stringent criteria (see Supplementary Table 8).

There is prior evidence for the genes involved in these three colocalization relationships being related to aggressive behavior. Cbln2 (cerebellin-2) is functionally associated with aggressive behavior, as shown by hyper aggressivity of Cbln2 knockout mice[54] and this behavioral phenotype has been linked to dysfunction of the serotonergic system. Pak3 (protein-activated kinase 3) has been bioinformatically linked to aggressive behavior in humans, and patients with PAK3 mutations have been reported to exhibit aggressive behavior[55]. The Irs4 (insulin receptor substrate 4) gene is known to control maternal aggression and Irs4 knockout female mice were found to be less aggressive to intruders[56]. Glra3 (glycine receptor alpha 3) has been associated with aggression-related phenotypes in GWAS studies[57].

While there is limited knowledge regarding reasons for their observed colocalization, there is in fact substantial evidence that Pak3 (involved in two of the three gene pairs) is locally translated in specific subcellular regions, suggesting a likely explanation for its colocalization with other functionally related genes.

PAK3 protein is localized to dendrites and colocalizes with PAK1 in dendritic spines[58], regulates dendritic spine formation[59], and has important roles in synaptic transmission and plasticity[60,61]. Moreover, Pak3 mRNA has also been found to be enriched in dendrites compared to somata[62], suggesting local synthesis of the protein for its dendritic functions.

The CBLN2 protein is also localized in dendrites and involved in dendritic spine formation[63], and, like other cerebellins, critically contributes to synapse formation[64]. While there is no direct experimental record of sub-cellular distribution of Cbln2 mRNA, its protein localization in dendrites and the general recognition of local translation during dendrite morphogenesis and synaptic plasticity[65] leaves open the possibility that this mRNA exhibits at least some level of dendrite localization.

Thus, our observation of Cbln2-Pak3 mRNA colocalization may be pointing to their respective tendencies to localize in dendrites and other cellular compartments, as part of the functional roles. The fact that this is the single most significant *differential* colocalization associated with males aggression-versus-naïve contrast further suggests that the recorded functions of the corresponding proteins in aggression (see above) may manifest at the sub-cellular level through a change in localization patterns and local translation rates.

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