Integrating barcoded neuroanatomy with spatial transcriptional profiling enables identification of gene correlates of projections

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Functional circuits consist of neurons with diverse axonal projections and gene expression. Understanding the molecular signature of projections requires high-throughput interrogation of both gene expression and projections to multiple targets in the same cells at cellular resolution, which is difficult to achieve using current technology. Here, we introduce BARseq2, a technique that simultaneously maps projections and detects multiplexed gene expression by in situ sequencing. We determined the expression of cadherins and cell-type markers in 29,933 cells and the projections of 3,164 cells in both the mouse motor cortex and auditory cortex. Associating gene expression and projections in 1,349 neurons revealed shared cadherin signatures of homologous projections across the two cortical areas. These cadherins were enriched across multiple branches of the transcriptomic taxonomy. By correlating multigene expression and projections to many targets in single neurons with high throughput, BARseq2 provides a potential path to uncovering the molecular logic underlying neuronal circuits.

Neural circuits are composed of neurons diverse in many properties, such as morphology1–4, gene expression3,4 and projections3,5. Although recent technological advances have made it possible to characterize the diversity in individual neuronal properties, associating multiple properties in single neurons with high throughput remains difficult to achieve. Investigating the relationship between multiple neuronal properties is essential for understanding the complex organization of neural circuits.

Of particular interest is the relationship between endogenous gene expression and long-range projections in the cortex. Cortical neurons have diverse patterns of long-range projections5,6 and diverse patterns of gene expression3,4. The full diversity of neuronal projection patterns can often only be appreciated by assessing multiple projection targets simultaneously (Fig. 1a)3,5. For example, Han et al. showed that neurons in mouse visual area V1 that project to area PM tend not to project to area AL and vice versa, a projection ‘motif’ that involves the relative probability that a single neuron projects to two targets and hence could not have been discovered by assessing projection targets one at a time. Gene expression patterns are also complex, and although the diversity in gene expression can be described by clustering neurons into transcriptomic types, these transcriptomic types have limited power in explaining the diversity of cortical projections beyond the major classes of projection neurons5,6,7. Moreover, because the determination of a transcriptomic type relies on the expression of only a subset of genes, the inability of transcriptomic type to predict projection patterns raises the possibility that the expression of other genes—potentially in gene coexpression motifs—might be better correlated with projection patterns. Although transcriptomic methods can be combined with retrograde labeling8,9, retrograde labeling is limited to one or at most a few brain areas at a time. Resolving the relationship between gene expression and projection patterns in the adult cortex thus requires high-throughput techniques that allow simultaneous multiplexed gene detection with projection mapping to multiple target areas at single-neuron resolution, which remains difficult to achieve.

To achieve high-throughput mapping of projections to many brain areas, we recently introduced barcoded anatomy resolved by sequencing (BARseq), a projection mapping technique based on in situ sequencing of RNA barcodes6. In BARseq, each neuron is labeled with a unique virally encoded RNA barcode that is replicated in the somas and transported to the axon terminals. The barcodes at the axon terminals located at various target areas are sequenced and matched to somatic barcodes, which are sequenced in situ, to determine the projection patterns of each labeled neuron. Because BARseq preserves the location of somata with high spatial resolution, in principle it provides a platform to combine projection mapping with other neuronal properties also interrogated in situ, including gene expression. We have previously shown that BARseq can be combined with fluorescence in situ hybridization (FISH) and Cre labeling to uncover projections across neuronal subtypes defined by gene expression. However, these approaches can only interrogate one or a few genes at a time, which would be insufficient for unraveling the complex relationship between the expression of many genes to diverse cortical projections (Fig. 1a).

Here we aim to develop a technique to simultaneously map projections to multiple brain areas and detect the expression of dozens of genes in hundreds to thousands of neurons from a cortical area with high throughput, high spatial resolution and cellular resolution. To achieve this goal, we combine the high-throughput and multiplexed projection mapping capability of BARseq with state-of-the-art spatial transcriptomic techniques with high imaging throughput and multiplexing capacity10,11. This second-generation BARseq (BARseq2) greatly improves the ability to correlate the expression of many genes to projections to many targets in the same neurons. As a proof of principle, we first demonstrate multiplexed gene detection using BARseq2 by mapping the spatial pattern of up
Fig. 1 | In situ sequencing of endogenous mRNAs using BARseq2. a. Cartoon of an example model in which the relationship between projections and gene expression can only be correctly inferred by multiplexed interrogation of both projections and gene expression. In this model, neurons that express both genes project to both targets A and B, whereas neurons that express only one of the two genes project randomly to either A or B, but not both. Methods that combine multiplexed single-neuron gene expression with data about only a single projection target will conclude that all three gene expression patterns project to target A, thus failing to detect the underlying ‘true’ relationship between gene expression and projections. Similarly, methods that combine multiplexed single-neuron projections with data about only a single gene will also fail to detect any relationship between gene expression and projections. b,c. BARseq2 correlates projections and gene expression at cellular resolution (b). In BARseq2, neurons are barcoded with random RNA sequences to allow projection mapping, and genes are also sequenced in the same barcoded neurons. RNA barcodes and genes are amplified and read out using different strategies (c). d, Theoretical imaging cycles using combinatorial coding (BARseq2), four-channel sequential coding or four-channel sparse coding as used by Eng et al.50. Imaging cycles assumed three additional cycles for BARseq2, one additional round for sparse coding, and no extra cycle for sequential coding for error correction. e, Mean and individual data points of the relative sensitivity of BARseq2 in detecting the indicated genes using different numbers of padlock probes per gene. The sensitivity is normalized to that using one probe per gene. n = 2 slices for each gene. f, Representative images of BARseq2 detection of the indicated genes using the maximum number of probes shown in e compared to RNAscope. Scale bars, 10 μm.

to 65 cadherins and cell-type markers in 29,933 cells. We then correlate the expression of 20 cadherins to projections to 35 target areas in 1,349 neurons in mouse motor and auditory cortex. Our study reveals new sets of cadherins that correlate with homologous projections in both cortical areas. BARseq2 thus bridges transcriptomic signatures obtained through spatial transcriptional profiling with sequencing-based projection mapping to illuminate the molecular logic of long-range projections.

Results
To investigate how cadherin expression relates to diverse projections, we developed BARseq2 to combine high-throughput projection mapping with multiplexed detection of gene expression using in situ sequencing (Fig. 1b,c). BARseq2 is based on BARseq (Fig. 1c), which achieves high-throughput projection mapping by in situ sequencing of RNA barcodes6. Projection patterns observed using BARseq are consistent with those obtained using conventional neuroanatomical techniques in multiple circuits5–7, but it can achieve throughput that is at least two to three orders of magnitude higher than the state-of-the-art single-cell tracing techniques8. Possible technical concerns, including distinguishing fibers of passage from axonal termini, sensitivity, double labeling of neurons and degenerate barcodes, have previously been addressed5,6,12,13 and will not be discussed in detail again here. Combining barcoded single-cell projection mapping with in situ detection of endogenous mRNAs exploits the unique advantage of BARseq in throughput to efficiently interrogate both neuronal gene expression and long-range projections simultaneously.

To detect gene expression using BARseq2, we used a non-gap-filled padlock probe-based approach to amplify target endogenous mRNAs10,11 (Fig. 1c). The elimination of gap filling, necessary for reading out extremely diverse sequences of barcodes, increases the sensitivity for endogenous gene detection. In this approach, the identity of the target is read out by sequencing a gene-identification index (GII) using Illumina sequencing chemistry in situ. Because the GII is a nucleotide barcode sequence that uniquely encodes the identity of a given gene, the multiplexing capacity increases exponentially as 4^N, where N is the number of sequencing cycles. This combinatorial coding by sequencing readout thereby allows simultaneous detection of a large number of genes using only a few cycles of imaging (Fig. 1d). Although sequencing readout offers many advantages, BARseq2 is also compatible with hybridization-based
readout when necessary. The combination of non-gap-filling in situ sequencing of endogenous genes and the gap-filling approach for sequencing barcodes allows many genes to be detected simultaneously with projections using BARseq2.

We first demonstrate that, by optimizing targeted in situ sequencing, BARseq2 could achieve sufficient sensitivity for detection of endogenous mRNAs. We next combined in situ sequencing of endogenous mRNAs with in situ sequencing of RNA barcodes to associate the expression of cadherins with projection patterns at cellular resolution. We then validated BARseq2 by demonstrating that it could be used to recapitulate projection patterns specific to transcriptional neuronal subtypes and to identify cadherins that were differentially expressed across major projection classes. Finally, we identified a set of cadherins shared between the mouse auditory cortex and motor cortex that correlate with homologous projections of intratelencephalic (IT) neurons in both cortical areas.

BARseq2 robustly detects endogenous mRNAs. To adequately detect genes using BARseq2, we sought to improve the detection sensitivity. In most in situ hybridization (ISH) methods, high sensitivity is achieved by using many probes for each target mRNA. We reasoned that increasing the number of padlock probes for each gene might similarly improve the sensitivity of BARseq2. Indeed, we observed that tiling the whole gene with additional probes resulted in as much as a 46-fold increase in sensitivity compared to using a single probe (Fig. 1c and Methods). Combined with other technical optimizations (Extended Data Fig. 1a,b), we increased the sensitivity of BARseq2 to 60% of RNAscope, a sensitive and commercially available FISH method (Fig. 1f, Extended Data Fig. 1c,d and Methods). We further optimized in situ sequencing to robustly read out GIs of single rolonies over many sequencing cycles (Extended Data Fig. 1e–j and Methods). These optimizations allowed BARseq2 to achieve sufficiently sensitive, fast and robust detection of mRNAs.

BARseq2 allows multiplexed detection of mRNAs in situ. To assess multiplexed detection of cadherins in situ using BARseq2, we examined the expression of 20 cadherins, along with either 3 (in auditory cortex) or 45 (in motor cortex) cell-type markers (Fig. 2a–c). We chose to focus on the cadherins because of their known roles in cortical development, including projection specification, and their differential expression among cardinal cell types defined by multiple properties. These cadherins included most classical cadherins and nonclustered protocadherins expressed in auditory cortex and motor cortex. We successfully resolved and decoded 419,724 rolonies from two slices of mouse auditory cortex (1.7 mm² × 10 μm per slice) and 1,445,648 rolonies from four slices of primary motor cortex (2.8 mm² × 10 μm per slice). We recovered 20 rolonies in auditory cortex and 115 rolonies in motor cortex matching two GIs that were not used in the experiment, corresponding to an estimated error rate of 0.1% and 0.2%, respectively, for rolony decoding.

Consistent with previous reports, many cadherins were enriched in specific layers and sublayers in the cortex (Fig. 2d). Interestingly, although most cadherins had similar laminar expression in both auditory cortex and motor cortex, some cadherins were differentially expressed across the two areas. For example, Cdh9 and Cdh13 were enriched in L2/3 in auditory cortex, but not in motor cortex (Fig. 2d and Extended Data Fig. 2). The laminar positions of peak cadherin expression were consistent with those obtained by other methods, including RNAscope (Fig. 2e) and the Allen Brain Atlas (ABA) database of ISH (Fig. 2f, Extended Data Fig. 3 and Methods). Thus, BARseq2 accurately resolved the laminar expression patterns of cadherins.

We then characterized gene expression obtained by BARseq2 at single-cell resolution (Methods). We assigned 228,371 rolonies to 3,377 excitatory or inhibitory neurons (67.6 ± 28.8 (mean ± s.d.) rolonies per neuron) in auditory cortex, and 752,687 rolonies to 11,492 excitatory or inhibitory neurons (65.5 ± 26.0 (mean ± s.d.) rolonies per neuron) in motor cortex. Most cadherins showed slight differences in single-cell expression levels in these two cortical areas (Extended Data Fig. 4). In auditory cortex, the total read counts per cell was higher in BARseq2 than in single-cell RNA sequencing (scRNA-seq) using 10x Genomics v3 (Fig. 2g; median read count was 64 for BARseq2 (n = 3,337 cells) compared to 57 for scRNA-seq (n = 640 cells); P = 5.3 × 10^−8, rank-sum test). Thus, even using a limited number of probes, BARseq2 achieved sensitivity at least equal to scRNA-seq using 10x v3. For experiments requiring better quantification of genes present at low expression, the sensitivity could potentially be further improved by using more probes.

Further analyses showed that detection of mRNA by BARseq2 was specific. The mean expression of genes determined by BARseq2 was highly correlated with that determined by scRNA-seq using 10x v3 (Fig. 2h; Pearson correlation r = 0.88). A few outliers had substantially more counts in BARseq2 than in scRNA-seq, likely reflecting sampling differences across cell types, area-specific gene expression and differences in RNA accessibility in situ. For example, Cdh6 expression observed by BARseq2 was 26 times that observed by scRNA-seq. This difference could be attributed to under-sampling of Cdh6-expressing pyramidal tract (PT) neurons in our scRNA-seq data and potentially variable sampling of neighboring cortical areas in which Cdh6 is differentially expressed.

Furthermore, correlations between pairs of genes in single neurons determined by BARseq2 were consistent with scRNA-seq using 10x v3 to a similar extent as two independent 10x v3 experiments (Fig. 2i–k, Extended Data Fig. 5a,b and Methods). These results indicate that the single-cell gene expression patterns observed by BARseq2 were comparable to those of scRNA-seq.

We wondered if BARseq2 could detect more genes in parallel, and thus be potentially useful in associating projections with larger...
gene panels. Because BARseq2 imaging time scales logarithmically with the number of genes detected (Fig. 1d), the multiplexing capacity of BARseq2 is not limited by imaging time. Furthermore, targeting up to 65 genes did not significantly affect the detection sensitivity of each gene (Extended Data Fig. 5c and Methods). The detection of this 65-gene panel in motor cortex (Fig. 3a) allowed us to classify neurons to one of nine transcriptomic neuronal types defined by scRNA-seq\(^1\) (Fig. 3b, Methods and Extended Data Fig. 5d–h). Consistent with previous studies\(^9\), these transcriptomic neuronal types displayed distinct laminar distributions (Fig. 3b,c and Methods) and cadherin expression (Fig. 3d). Most transcriptomic types were found in the expected layers with the notable exception of L5 PT and L6 IT Car3, which were seen in additional layers (for example, L2/3). These inaccuracies in cell typing likely...
resulted from suboptimal choice of marker genes (see Methods for a detailed discussion), and could potentially be improved in the future by optimizing the gene panels. These optimizations, however, were outside the scope of this study. These results demonstrate that BARseq2 can be applied to probe gene panels consisting of high dozens of genes, with minimal decrease in sensitivity and minimal increase in imaging time.

BARseq2 correlates gene expression to projections. Previous studies of the relationship between projection patterns and gene expression have largely focused on revealing the projection patterns of transcriptomic neuronal types. Although this approach has identified some projection patterns biased in certain transcriptomic types, the diversity of projections in IT neurons remains largely unexplained by transcriptomic types. To further understand the relationship between gene expression and projections, we demonstrate an alternative approach that screens a targeted panel of genes for correlates of diverse projections. This approach relies on the ability of BARseq2 to interrogate both the expression of many genes and projections to many targets simultaneously, and thus would have been difficult to achieve using existing transcriptomic approaches that could only interrogate one or a small number of genes.
As a proof-of-principle study, we examined long-range axonal projections and the expression of 20 cadherins, along with three marker genes, in motor cortex and auditory cortex in three mice. We optimized BARseq2 to detect both endogenous mRNAs and barcodes in the same barcoded neurons without compromising sensitivity (Extended Data Fig. 6a and Methods). In each barcoded cell, we segmented barcoded cell bodies using the barcode sequencing images (Fig. 4a). We then assigned rolonies amplified from endogenous genes that overlapped with these pixels to the barcoded cells (Fig. 4a). This allowed us to map both projection patterns and gene expression (Fig. 4b) in the same neurons. We matched barcodes in these target sites to 3,164 well-segmented barcoded neurons (1,283 from auditory cortex and 1,881 from motor cortex) from 15 slices of auditory cortex and 16 slices of motor cortex, each with 10-µm thickness. Of the barcoded neurons, 624 and 791 neurons had projections above the noise floor in auditory cortex and motor cortex, respectively. Most neurons (53% (329/624) in auditory and 89% (703/791) in motor cortex) projected to multiple brain areas. We then focused on 598 neurons in auditory cortex and 751 neurons in motor cortex, which also had sufficient endogenous mRNAs detected in each cell, for further analysis (Fig. 4c). These observations were largely consistent with previous BARseq experiments in auditory and motor cortex performed without assessing gene expression\(^2\), confirming that the modifications for BARseq2 did not compromise projection mapping.

BARseq2 recapitulates known projection biases. Although BARseq2 can read out gene expression and projections in the same neurons, one might be concerned that barcoding neurons using Sindbis virus could disrupt gene expression\(^6\). To determine the relationship between genes and projections, one would require that the gene–gene relationship in Sindbis-infected single neurons reflects that in noninfected neurons, and that any change in absolute gene expression level would have little effect. Reassuringly, previous reports have shown that the relationship among genes in single neurons is indeed largely preserved despite a reduction in the absolute expression of genes in Sindbis-infected cells\(^2\). Furthermore, correlations between transcriptomic types and projections revealed in Sindbis-infected neurons were corroborated by other methods that did not require Sindbis infection\(^2\). In agreement with these previous reports, we observed that the correlations between pairs of genes in the barcoded neurons were consistent with those in non-barcoded neurons despite an overall reduction in gene expression (Extended Data Fig. 6b–f and Methods). Therefore, the relationship between gene expression and projections resolved by BARseq2 likely reflects that in non-barcoded neurons.

To further test whether BARseq2 can capture the relationship between gene expression and projections, we asked if we could identify differences in projection patterns across transcriptomic neuronal types that could also be validated by previous studies and/or other experimental techniques. We performed these validation analyses at three different levels of granularity. First, BARseq2 confirmed that most barcoded neurons with long-range projections were excitatory, not inhibitory; whereas about 8–9% of all barcoded neurons were inhibitory (100 of 1,047 in auditory cortex and 140 of 1,689 in motor cortex; Fig. 4d), only 7 of 240 (3%) inhibitory neurons (5 in auditory cortex and 2 in motor cortex) had detectable projections (Fig. 4c, Methods and Extended Data Fig. 6g,h). Second, BARseq2 identified many cadherins (8 for auditory cortex and 12 for motor cortex) that were differentially expressed across IT, PT and corticothalamic (CT) neurons\(^7\) (Fig. 5a–d); the differential expression of these genes was consistent with the expression observed by scRNA-seq\(^2\) (Extended Data Fig. 7a and Methods). Finally, BARseq2 confirmed known biases in projection patterns across transcriptionally defined IT subtypes in auditory cortex (Extended Data Fig. 7b,c and Methods). Thus, BARseq2 recapitulated known projection differences across transcriptomic subtypes of IT neurons.

BARseq2 identifies cadherin correlates of IT projections. Having established that BARseq2 identified gene correlates of projections that were consistent with previous studies, we then asked whether cadherin expression correlates with projection patterns within the IT class of neurons. Although cadherins and other cell adhesion molecules are involved in projection specification and axonal growth during development\(^2\), many take on other functions unrelated to projection specification in later developmental stages\(^2\). In addition, other mechanisms such as axonal pruning could further shape the projection patterns of neurons independent of initial genetic programs. Therefore, any correlation between cadherins and projections is likely a remnant, or ‘echo,’ of the developmental program that initially specified projections, and may thus be weak and further obscured by gene expression associated with later developmental stages. To overcome the challenges of identifying potentially weak relationships between gene expression and projections, we used BARseq2 to identify correlations between projections and cadherins using a module-based strategy inspired by similar approaches in transcriptomics\(^8\). Projection modules and gene modules average over the noise in the measurement of individual projections and genes, respectively, and are thus easier to detect when there is considerable biological and/or technical noise in the measurements. This approach requires knowing the projections to many brain areas from individual neurons, a unique advantage of barcoding-based projection mapping techniques (that is, BARseq and BARseq2) compared to retrograde labeling-based approaches\(^9\). Next, we identify modest associations between cadherin expression and projections in IT neurons, including several associated pairs of cadherins/projections that were shared across cortical areas.

The projections of an IT neuron to its targets are not random. Rather, in both auditory cortex and motor cortex, these projections are organized and show statistical regularities that can be uncovered within the large datasets obtained by BARseq\(^2\) (Fig. 6a). For example, neurons in the auditory cortex that projected to the somatosensory cortex were also more likely to project to the ipsilateral visual cortex, but not the contralateral auditory cortex. To
exploit these correlations, we used nonnegative matrix factorization (NMF)\(^2\) to represent the projection pattern of each neuron as the sum of several 'projection modules.' (NMF is an algorithm related to principal-component analysis, but imposes the added constraint that projections are nonnegative). Each of these modules (six modules for the motor cortex and three for the auditory cortex; Fig. 6b)
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consisted of subsets of projections that were likely to co-occur. We named these modules by the main projections (cortex (CTX) or striatum (STR)) followed by the side of the projection (ipsilateral (I) or contralateral (C)). For some modules, we further indicated that the projections were to the caudal part of the structure by prefixing with ‘C’ (for example, CSTR-I or CCTX-I). A small number of projection modules could explain most of the variance in projections (three modules and six modules explained 84% and 87% of the variance in projections to nine areas in auditory cortex and 18 areas in motor cortex that IT neurons project to, respectively; Fig. 6c).

Because both the projection patterns of neurons27 and their transcriptomic types are well correlated with laminae, we first asked how well cadherins explained the diversity of projections in IT neurons compared to the laminar positions of neurons (Methods). Although most cadherins had no predictive power on the projection modules, some individual cadherins could explain a substantial fraction of the variance in projections compared to that explained by the laminar positions of neurons (Extended Data Fig. 8). For example, Cdh13 and Pcdh7 explained 6.0% ± 0.3% and 7.0% ± 0.3% (mean ± s.d.) of the variations in CTX-C in auditory cortex, compared to 19.4% ± 0.3% (mean ± s.d.) explained by the laminar positions of neurons. Strikingly, Pcdh19 and Pcdh7 were predictive of CSTR-I in auditory cortex, whereas the laminar positions were not. These results indicate that some but not all cadherins were modestly predictive of projections, and that the predictive power of these cadherins could be comparable in magnitude to the laminar positions of neurons, one of the strongest known predictors of projection patterns.

To further understand how cadherin expression relates to projections, we examined how it covaried with projection modules (Supplementary Fig. 1). Interestingly, the expression of several cadherins covaried with similar projection modules in both cortical areas. For example, auditory cortex neurons expressing Pcdh19 were stronger in the CSTR-I projection module than those not expressing Pcdh19 (Fig. 6d; \( P = 5 \times 10^{-4} \)) comparing the CSTR-I module in neurons with \((n = 83)\) or without \((n = 346)\) expression using rank-sum test); the same association between Pcdh19 and the CSTR-I projection module was also seen in motor cortex (Fig. 6d; \( P = 4 \times 10^{-4} \) using rank-sum test, \( n = 31 \) for Pcdh19+ neurons and \( n = 512 \) for Pcdh19− neurons). Similarly, Cdh8 was correlated with the CTX-I module and Cdh12 was correlated with the CTX-C module (Fig. 6e; false discovery rate (FDR) < 0.1) in both auditory and motor cortex. These correlations were independently validated by retrograde tracing using cholera toxin subunit B (CTB) and FISH (Extended Data Fig. 9a–e and Methods). Pcdh19, together with Cdh8 or Cdh11, correlated with CTX-I or CSTR-I modules, respectively, in motor cortex (Fig. 6e and Extended Data Fig. 8), consistent with a potential combinatorial nature of cadherin correlates of projections. Although the correlations between individual cadherins and projections were relatively modest, our observations that the same cadherins correlated with similar projection modules in both areas suggest that a common molecular logic might underscore the organization of projections across cortical areas beyond class-level divisions.

Analyses based on the expression of single genes suffer from biological and technical noise of gene expression in single neurons. We reasoned that the correlations among genes might allow us to identify additional relationships between gene expression and projections that were missed by analyzing each gene separately. This ability to leverage the relationship among genes represents an advantage of BARseq2 over the original BARseq because of the improved capacity of BARseq2 for multiplexed gene detection. To exploit the correlations among genes, we grouped 16 cadherins into three meta-analytic coexpression modules based on seven scRNA-seq datasets of IT neurons in motor cortex (Fig. 7a and Extended Data Fig. 10a,b)23. To obtain the modules, we followed the rank-based network aggregation procedure defined by Ballouz et al.33 and Crow et al.24 to combine the seven dataset-specific gene–gene coexpression networks into an aggregated network, and then grouped together genes showing consistent excess correlation using the dynamic tree-cutting algorithm. Two coexpressed modules were associated with projections: module 1 was associated with contralateral striatal projections (STR-C projection module), and module 2 was associated with ipsilateral caudal striatal projections (CSTR-I; Fig. 7b,c and Extended Data Fig. 10c,d). These associations between the coexpression modules and projections were consistent with, but stronger than, associations between individual genes contained in each module and the same projections (Extended Data Fig. 10e). Interestingly, these coexpression modules were enriched in multiple transcriptomic subtypes of IT neurons, but these transcriptomic subtypes were found in multiple branches of the transcriptomic taxonomy (Fig. 7d and Extended Data Fig. 10f). For example, module 1 was associated with transcriptomic subtypes of IT neurons in L2/3, L5 and L6. This result is consistent with previous observations that first-tier transcriptomic subtypes of IT neurons (that is, subtypes of the highest level in the transcriptomic taxonomy within the IT class) appeared to share projection patterns, and further raises the possibility that transcriptomic taxonomy does not necessarily capture differences in projections. Taken together, our finding that projections correlate with cadherin coexpression modules independent of transcriptomic subtypes demonstrates that BARseq2 can reveal intricate relationships between gene expression and projection patterns.

Discussion

BARseq2 combines high-throughput mapping of projections to many brain areas with multiplexed detection of gene expression at single-cell resolution. Because BARseq2 is high throughput, we are able to correlate gene expression and projection patterns of thousands of individual neurons in a single experiment, and thereby achieve statistical power that would be challenging to obtain using other single-cell techniques. By applying BARseq2 to two distant cortical areas—primary motor and auditory cortex—in the adult mouse, we identified cadherin correlates of diverse projections. Our results suggest that BARseq2 provides a path to discovering...
the general organization of gene expression and projections that are shared across the cortex.

**High-throughput and multiplexed gene detection by BARseq2.**

To correlate panels of genes to projections, we designed BARseq2 to detect gene expression with high throughput, for multiplexing to dozens of genes, to have sufficient sensitivity, and be compatible with barcoding-based projection mapping. To satisfy these needs, we based BARseq2 on padlock probe-based approaches. With additional optimizations for sensitivity, sequencing readout...
Cadherins correlate with diverse projections of IT neurons. As a proof-of-principle study, we used BARseq2 to identify several cadherins that correlate with homologous IT projections in both auditory and motor cortex, two spatially and transcriptomically distant areas with distinct cortical and subcortical projection targets. In addition, cadherin coexpression modules that correlated with projections were associated with multiple branches of the transcriptomic taxonomy. This type of correlation between neuronal connectivity and variations in gene expression independent of transcriptomic types is not unique to the cortex and has previously been observed in other brain areas, such as the hippocampus. Therefore, our findings are consistent with the hypothesis that a shared cell adhesion molecule code might underlie the diversity of cortical projections independent of transcriptomic types.

Even though the power of some cadherins to predict projections was comparable in magnitude to that of laminar position, a strong predictor of projection patterns, these cadherins could only explain a small fraction of the overall variance in projections. This noisy association between cadherin expression and projection patterns contrasts with the known roles of cadherins in specifying neuronal connectivity in the cortex and other circuits, but the relatively small magnitude of these associations is not surprising for a few reasons. First, gene expression programs and signaling cues needed for specifying projections are usually transient in development, so it is likely that these cadherins only represent the remnants of labeling allows interrogation of the gene expression and projection patterns of a genetically defined subpopulation of neurons. However, this approach lacks cellular resolution, is limited by the availability of Cre lines, and requires that a neuronal population of interest be specifically distinguished by the expression of one or two genes. The combination of single-cell transcriptomic techniques with retrograde labeling does provide cellular resolution, but can only interrogate projections to one or at most a small number of brain areas at a time. The inability to interrogate projections to many brain areas from the same neuron would miss higher-order statistical structures in projections, which are nonrandom and provide additional information regarding other properties of the neurons, such as laminar position and gene expression. The projections of individual neurons to multiple brain areas can be obtained using multiplexed single-cell tracing, but the throughput of these methods remains relatively low. Moreover, many advanced single-cell tracing techniques require special sample processing that hinders multiplexed interrogation of gene expression in the same sample. The throughput of single-cell projection mapping was addressed by the original BARseq, but the small number of genes (up to three) that could be co-interrogated with projections limited its use in identifying the general relationship between gene expression and projections. BARseq2 thus addresses limitations of existing techniques and provides a powerful approach for probing the relationships between gene expression and projection patterns.

BARseq2 reveals gene correlates of projections. BARseq2 exploits the high-throughput axonal projection mapping that BARseq offers to identify gene correlates of diverse projections. BARseq has sensitivity comparable to single-neuron tracing. Although the spatial resolution of BARseq for projections is lower than that of conventional single-neuron tracing, it offers throughput that is several orders of magnitude higher than the state-of-the-art single-cell tracing techniques. This high throughput allows BARseq to reveal higher-order statistical structure in projection patterns that would have been difficult to observe using existing techniques, such as single-cell tracing. The increased statistical power of BARseq, obtained at the cost of some spatial resolution, is reminiscent of different clustering power across scRNA-seq techniques of varying throughput and read depth. The high throughput of BARseq thereby provides a powerful asset for investigating the organization of projection patterns and their relationship to gene expression.

BARseq2 enables simultaneous measurement of multiplexed gene expression and axonal projections to many brain areas, at single-neuron resolution and at a scale that would be difficult to achieve with other approaches. For example, Cre-dependent
a common developmental program that establish projections\(^2\), or may be needed for ongoing functions or maintenance of projections. Second, non-cadherin cell adhesion molecules (for example, IgCAMs\(^3,4\)) and other cell-surface molecules (for example, plexins, semaphorins\(^5\) and teneurins\(^6\)) are also involved in specifying projections, so cadherins likely only represent a fraction of the molecular programs that specify projections. Finally, cortical projections undergo extensive activity-dependent modifications after the initial specification, so the overall diversity in cortical projections is likely much higher than that produced by the initial molecular program. These possibilities can be better resolved by applying BARseq2 to reveal gene expression in both the projection neurons and the areas they project to during development, in combination with perturbation experiments. BARseq2 thus provides a path to discovering
the myriad of genetic programs that specify and/or correlate with long-range projections in both developing and mature animals.

**BARseq2 builds a unified description of neuronal diversity.**

Neuronal barcoding was originally proposed as a method for untangling circuit connectivity at synaptic resolution\(^4\).\(^5\). Solving neuronal connectivity with barcode sequencing not only has the potential to achieve high-throughput and single-cell resolution by exploiting advances in sequencing technology, but also provides a path to integrate measurements of multiple neuronal properties in single neurons—toward the ‘Rosetta brain’\(^6\). BARseq2 is a step toward this goal. Although BARseq2 currently only resolves projections at relatively low spatial resolution (brain areas, that is hundreds of microns), this limitation can be addressed in the future by using in situ sequencing to read out axonal barcodes (Yuan et al., unpublished data), which would resolve axonal projections at subcellular spatial resolution. Further combining in situ sequencing of neuronal properties using spatial information, it is potentially compatible with other in situ assays, such as immunohistochemistry, two-photon calcium imaging and dendritic morphological reconstruction. By spatially correlating various neuronal properties in single neurons, BARseq2 represents a feasible path toward achieving a comprehensive description of neuronal circuits.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at [https://doi.org/10.1038/s41593-021-00842-4](https://doi.org/10.1038/s41593-021-00842-4).

Received: 25 August 2020; Accepted: 19 March 2021; Published online: 10 May 2021

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Methods

Animal processing and tissue preparation. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee (protocol no. 19-16-10-07-03-00-4) at Cold Spring Harbor Laboratory. The animals were housed at maximum of five in a cage on a 12-h on/12-h off light cycle. The temperature in the facility was kept at 22 °C with a range not exceeding 20.5 °C to 26 °C. Humidity was maintained at around 45–55%, not exceeding a range of 30–70%. A list of animals used is provided in Supplementary Table 1.

For samples used only for endogenous mRNA detection, 8- to 10-week-old male C57BL/6 mice were anesthetized and decapitated. We immediately added the brain in optimal cutting temperature (OCT) compound in a 22-mm cryomold and snap froze the tissue in an isopentane bath submersed in liquid nitrogen. Sections were cut into 10-µm-thick slices on SuperFrost Plus Gold Slides (Electron Microscopy Sciences). Unlike in the original BARseq, the sections were directly melted onto slides without the use of a tape-transfer system. This change in mounting methods allowed increased efficiency in gene detection. The slides were stored at −80 °C until use.

For BARseq2 samples, 8- to 10-week-old male C57BL/6 mice were injected as indicated in Supplementary Table 1. After 24 h, we anesthetized and decapitated the animal, punched out the injection site and snap froze the rest of the brain on a razor blade on dry ice for conventional MAPseq. The injection site was embedded, cryosectioned and stored as described above.

To prepare samples for BARseq experiments, we immersed slides from −80 °C instantly into freshly made 4% paraformaldehyde (10-mL vials of 20% PFA; Electron Microscopy Sciences) in PBS for 30 min at room temperature. We washed the samples in 0.2 %BS(PEG)9 for 1 h, neutralized with 1 M Tris-HCl at pH 8.0 for 30 min, and then washed the sample with PBST twice. After reverse transcription, we washed the samples in the reverse transcription mixture at 37 °C overnight. After reverse transcription, we ligated the probe for the barcode cDNA (100 mM barcode padlock (IDT), 50 µM dNTPs, 5% glycerol, 1 U µl−1 Ribolock RNase Inhibitor, 20% formamide (Thermo Fisher Scientific), 50 mM KCl, 0.4 U µl−1 Ampligase (Epicentre) in 1X Ampligase buffer) for 30 min at 37 °C and 45 min at 45 °C. After ligation padlock probes for our target genes, we ligated the probe for the barcode cDNA (100 mM barcode padlock (IDT), 50 µM dNTPs, 5% glycerol, 1 U µl−1 Ribolock RNase Inhibitor, 20% formamide (Thermo Fisher Scientific), 50 mM KCl, 0.4 U µl−1 RNase H (Qiagen), 0.001 U µl−1 Phusion DNA polymerase (NEB) and 0.5 U µl−1 Ampligase (Epicentre) in 1X Ampligase buffer) for 10 min at room temperature. After RCA, we again cross-linked the rolonies in 0.25-mm; Grace Bio-Labs) for subsequent reactions on the sample. We then dehydrated the samples in 70%, 85% and 100% ethanol for 5 min each, followed by washing in 100% ethanol for at least 1 h at 4 °C. Finally, we rehydrated the samples in PBST (0.5% Tween-20 in PBS).

For retrograde labeling experiments, we prepared 1.0 mg ml−1 of CTB in PBS from 100mg for injections (see Supplementary Table 1 for a list of animals and coordinates used). We perfused the animals with fresh 4% PFA 96 h after injection, post-fixed for 24 h in 4% PFA, and cryoprotected in 10% sucrose in PBS for 12 h, overnight at room temperature. After RCA, we again cross-linked the rolonies in 0.2 %BS(PEG)9 for 1 h, neutralized with 1 M Tris-HCl at pH 8.0 for 30 min, and then washed the sample with PBST twice. After reverse transcription, we washed the samples in the reverse transcription mixture at 37 °C overnight.

BARseq2 detection of endogenous genes. We prepared a master mix of reverse transcription primers at 0.5 µM each for all target mRNAs. For volumes exceeding the amount required for reverse transcription, we speed-vacuum concentrated the primer mix into a smaller volume. We then prepared the reaction (0.5 µg per gene of RT primer (IDT), 1 U µl−1 RiboLock RNA inhibitor (Thermo Fisher Scientific), 0.2 µg µl−1 BSA, 500 µM dNTPs (Thermo Fisher Scientific), 20 U µl−1 RevertAid H-Minus M-MuLV reverse transcriptase (Thermo Fisher Scientific) in 1X RT buffer). We incubated the samples in the reverse transcription mixture at 37 °C overnight. After reverse transcription, we cross-linked the cDNAs in 50 mM BS(PEG)9 for 1 h and neutralized excess cross-linker with 1X HCl at pH 8.0 for 30 min, and then washed the sample with PBST twice to eliminate excess Tris buffer. We then prepared a master padlock mix with 200 nM per padlock probe for each target mRNA and speed-vacuum concentrated the amount required for reverse transcription, we speed-vacuum concentrated the primer mix into a smaller volume. We then prepared the reaction (0.5 µg per gene of RT primer (IDT), 1 U µl−1 RiboLock RNA inhibitor (Thermo Fisher Scientific), 0.2 µg µl−1 BSA, 500 µM dNTPs, 5% glycerol and 1 U µl−1 RiboLock RNase Inhibitor, 20% formamide (Thermo Fisher Scientific), 50 mM KCl, 0.4 U µl−1 Ampligase (Epicentre) in 1X Ampligase buffer) for 30 min at 37 °C and 45 min at 45 °C. After ligation padlock probes for our target genes, we ligated the probe for the barcode cDNA (100 mM barcode padlock (IDT), 50 µM dNTPs, 5% glycerol, 1 U µl−1 Ribolock RNase Inhibitor, 20% formamide (Thermo Fisher Scientific), 50 mM KCl, 0.4 U µl−1 RNase H (Qiagen), 0.001 U µl−1 Phusion DNA polymerase (NEB) and 0.5 U µl−1 Ampligase (Epicentre) in 1X Ampligase buffer) for 10 min at room temperature. After RCA, we again cross-linked the rolonies in 0.2 %BS(PEG), 50 µM dNTPs, 5% glycerol and 1 U µl−1 q29 DNA polymerase (Thermo Fisher Scientific) in 1X q29 DNA polymerase buffer) overnight at room temperature. After RCA, we again cross-linked the rolonies in 0.2 %BS(PEG) for 1 h, neutralized with 1 M Tris-HCl at pH 8.0 for 30 min, and washed with PBST. We then washed the sample in PBST twice, incubated in 125 µM amido-allyl dUTP (Thermo Fisher Scientific), 20 U µl−1 Ampligase (Epicentre) in 1X Ampligase buffer) for 30 min at 37 °C and 45 min at 45 °C. Finally, we performed rolling circle amplification (RCA; 125 µM amido-allyl dUTP (Thermo Fisher Scientific), 29 νl µl−1 CTB in PBS (ACDBio), 1 U µl−1 RiboLock RNase inhibitor (Thermo Fisher Scientific), 100 µl µl−1 BSA, 250 µM dNTPs, 5% glycerol and 1 U µl−1 q29 DNA polymerase (Thermo Fisher Scientific) in 1X q29 DNA polymerase buffer) overnight at room temperature.

In situ sequencing of endogenous genes. To sequence the endogenous genes using Illumina sequencing chemistry, we used the HiSeq Rapid SBS Kit v2 reagents to reduce cost from the original sequencing protocol. For the first cycle, we incubated samples in universal sequencing buffer (USB) at 60 °C for 3 min, then washed in PBST, followed by incubation in iodoacetamide (9.3 mg in 2 ml PBST) at 60 °C for 3 min. We then washed the sample in PBST again, rinsed with USB twice more, and then incubated in incorporation mix (IRM) at 60 °C for 3 min. We repeated the IRM step again to ensure the reaction was as close to 100% complete as possible. We then washed the sample in PBST once and then continued to wash in PBST four more times at 60 °C for 3 min each time. To reduce bleeding during imaging, we imaged the sample in universal scan mix (USM) for 2 min each, we first washed samples in USB, then incubated in cleavage reagent master mix (CRM) at 60 °C for 3 min. We repeated the CRM step to ensure complete reaction and washed out residual CRM twice with cleavage wash mix (CWM). We then washed the sample with USB, and then with PBST, before incubating in iodoacetamide at 60 °C for 3 min. We repeated this step again to ensure we blocked as many of the free thiol groups as possible to reduce background. We then continued with IRM and PBST washes as described for the first cycle and imaged after each cycle. We performed four sequencing cycles and seven sequencing cycles in total for our cadherin panel of 23 genes and our motor cell-type markers and cadherin panel of 65 genes, respectively.

To visualize high expression, we cleaved the fluorophores in the last sequencing cycle and washed the sample with CWM and PBST. We then washed our sample in hybridization buffer and added probe detection solution (0.5 µM for each probe in hybridization buffer) for four different fluorescent probes detecting Slc17a7, Gad1, Slc30a3 and all previously sequenced genes, respectively, for 10 min at room temperature. We washed the sample in the same hybridization buffer three times for 2 min each, washed in PBST, then added DAPI stain (ACDBio) for 2 min at room temperature. We rinsed in PBST again and finally in USM for imaging.

In situ sequencing of barcodes. After sequencing and hybridizing for endogenous genes as described above, we stripped the sample of all hybridized oligonucleotides and sequenced bases by incubating twice in strip buffer (40% formamide in 2X SSC with 0.1% Triton-X) at 60 °C for 30 min. For master padlock (IDT) at 60 °C for 30 min. We then washed the sample with PBST, then washed with hybridization buffer, and then incubated samples in barcode sequencer hybridization solution (1µM sequencing primer in hybridization buffer) for 10 min at room temperature. We washed the sample with hybridization solution three times for 2 min each, before rinsing twice in PBST. We sequenced barcodes with the same sequencing procedure as described for endogenous genes but for 15 cycles in total. After 1 cycle or 5, we eliminated the iodoacetamide step. In the absence of the rest of sequencing because iodoacetamide blockage is irreversible, so further incubation in this block becomes unnecessary after several cycles.

Target area barcode sequencing. Barcode sequencing in target brain areas was performed for the Cold Spring Harbor Laboratory. For more following procedures used in a previous study. The target areas were dissected to match two other studies in A1 (ref. 4) and in M1 (ref. 7) resulting in 11 and 35 projection targets for neurons in auditory cortex and motor cortex, respectively; these projection targets corresponded to most of the major projection targets based on
The samples were then digested in Protease III for 30 min at 40 °C, and washed or drew a barrier around the samples using an ImmEdge hydrophobic barrier pen. The samples were then dehydrated in 50%, 75% and 100% ethanol twice for 5 min each. After air-drying the samples, we either mounted the samples with coverslips finally for imaging. We found that using multiple padlocks per mRNA transcript, with each padlock targeting a different site on the mRNA coding sequence, increased detection efficiency substantially (Fig. 1e). The increase in sensitivity varied across genes, but for short, highly expressed genes, almost all padlocks were able to detect expression of the same neurons without compromising sensitivity. We maximized the number of padlock probe sets for each gene so that we had at most 12 probe sets per gene. Some shorter genes had fewer than 12 probes. These panels resulted in sensitivity that was sufficient for the present experiments, albeit somewhat below the maximum achievable with more probes. All but three genes (Slc17a7, Slc30a3 and Gad1) were visualized using combinatorial GIID codes (4 nucleotides in auditory cortex and 7 nucleotides in motor cortex; Supplementary Table 4); only a small subset of the probes for each gene yielded signals high enough to enable visualization of expression patterns across single neurons. However, for the Gad1 gene, which we normalized the sensitivities. For tissue pretreatment, we found that thin-fresh-frozen tissue cryosections fixed with 4% PFA for 30 min to 1 h (Extended Data Fig. 1a) yielded higher mRNA sensitivity than shorter fixation or other pretreatments, such as PFA-perfused tissue slices with or without post-fixation. For reverse transcription, we found that reverse transcription primers specific to each probe set at a concentration of 5–50 µM each yielded the highest signal when using random primers at concentrations up to 50 µM (Extended Data Fig. 1b). Altogether, these optimizations were crucial for increased mRNA detection sensitivity comparable to hybridization-based techniques.

To multiplex gene detection with high imaging throughput, we optimized in situ sequencing to robustly read out GIs of single rolonies over many sequential cycles. We had previously adapted Illumina sequencing chemistry to sequence neuronal somata filled abundantly with RNA barcode rolonies, that is, DNA nanoballs generated by RCA,6,35. However, directly applying this method to sequence neuronal somata filled abundantly with RNA barcode rolonies, that is, DNA nanoballs generated by RCA,6,35. However, directly applying this method to sequence single rolonies generated from individual mRNAs proved difficult due to heating cycles and harsh stripping treatments that led to loss and/or jittering of rolonies (Extended Data Fig. 1e). To allow robust sequencing of single rolonies, we optimized cryosectioning and amino-allyl-dUTP concentration1 to cross-link rolonies more extensively, achieving less spatial jitter of single rolonies between imaging cycles (Extended Data Fig. 1e–h) and stronger signals (Extended Data Fig. 1i) retained over cycles. This robust in situ sequencing of combinatorial GIID codes allowed BARseq2 to achieve fast imaging critical for high-throughput correlation of gene expression with projections.

**Simultaneous detection of endogenous mRNAs and barcodes using BARseq2.** To assess multiplex gene expression and long-range projections in the same cells, we optimized for simultaneous detection and amplification of both endogenous mRNAs and barcodes. Although both endogenous mRNAs and barcodes are amplified using padlock probes, amplifying barcodes required the addition of a DNA polymerase to copy barcode sequences into padlock probes to allow direct sequencing of diverse barcodes (up to ~10^6 diversity; Fig. 1c). Directly combining the two processes reduced the detection sensitivity of target mRNAs due to the addition of the DNA polymerase (Extended Data Fig. 6a; 37% ± 3% (mean ± s.d., comparing the control condition to the zero-polymerase concentration). To preserve detection sensitivity for endogenous mRNAs while allowing the sequencing of diverse barcodes, we adjusted the concentration of the DNA polymerase to 0.001 U µl−1 (1/200 of the amount in the original BARseq), which doubled the sensitivity for endogenous mRNAs while also maintaining the sensitivity for barcodes (Extended Data Fig. 6a). This optimization allowed BARseq to detect both endogenous mRNAs and RNA barcodes together in the same neurons without compromising sensitivity.

**Single-cell RNA-seq of auditory cortex.** To dissociate neurons for scRNA-seq, we anesthetized animals with isoflurane and decapitated the animals. We then used a 2-mm biopsy punch to remove the auditory cortex. The tissue was then...
To find the barcode sequences of the segmented cell, we integrated signals over the whole segmented cells and called the channel with the strongest signal as the base in both the high-resolution images and the low-resolution images. We concatenated the high-resolution images and the low-resolution images to produce the full barcode sequences. To find the projection patterns, these in situ sequenced barcodes were then matched to the barcodes identified in the projection areas allowing one mismatch but not ambiguous matches (that is, one in situ barcode matching to multiple barcodes found in projection sites).

Analysis of BARseq2 gene expression data. All analyses were carried out in MATLAB. For analysis of gene-only datasets, neurons were first filtered by requiring at least ten counts of Slc17a7 or Gad1 and were positioned within the cortex. To make the data comparable to previous studies, the cortical depths of neurons were normalized using the thickness of 120 µm through 1,200 µm and 1,500 µm for motor cortex. To find cadherins that were differentially expressed in cell types, the expression of cadherins in each cell type was compared to the expression of cadherins in all other cell types using rank-sum tests.

Laminar distribution of cadherins. Because many genes, especially cell adhesion molecules, are differentially expressed across cortical layers, we evaluated how well BARseq2 can capture spatial organization of cadherins compared to existing methods, such as FISH. To compare laminar distribution observed by BARseq2, FISH and Allen Brain Atlas, we quantified gene expression signal densities across 100 µm bins in laminar depth. For BARseq2 and FISH, the quantification was performed by counting dots, for Allen Brain Atlas, the quantifications were done by integrating signal intensities over all pixels in each bin. Because each bin had a different number of pixels sampled in our data, we then divided the gene expression signals by the area observed in the images to calculate the density. We then z-scored the densities within each gene to produce the laminar profiles for each gene.

RNAseqcope against Cd8b, Pcdh19 and Pcdh20 revealed laminar expression profiles that were qualitatively similar to those obtained by BARseq2 (Fig. 2e). For Pcdh20, the dynamic range of gene expression (that is, the differences between peaks and valleys in expression) was more pronounced in the BARseq2 data than that observed by RNAseqcope. Because low sensitivity and/or low specificity would likely result in a reduction, not an increase, in the dynamic range of expression, it is unlikely that such quantitative differences in the laminar profiles of gene expression were caused by sensitivity and/or specificity issues with BARseq2. We suspect that the reduced dynamic range in RNAseqcope is caused by nonspecific signals inherent to amplified FISH methods. We therefore sought to compare BARseq2 to other FISH datasets to confirm its accuracy.

We then compared the distributions of genes obtained by BARseq2 to those in the Allen gene expression atlas21 (Fig. 2f and Extended Data Fig. 3). The laminar distribution of gene expression revealed by BARseq2 was highly correlated with that in the Allen gene expression atlas (Spearman correlation $\rho = 0.696, P = 3.8 \times 10^{-15}$). Specifically, the laminar distribution of Pcdh20 obtained by BARseq2 matched very well with Pcdh20 in the Allen gene expression atlas (Extended Data Fig. 3). These results indicate that BARseq2 accurately captured the laminar distribution of cadherin expression.

Gene-pair expression in single neurons. To test whether BARseq2 accurately captures gene expression, we compared the expression of two pairs of genes in single neurons. First, we compared the expression of Slc17a7 and Gad1, two genes that are expressed in two distinct classes of neurons. Second, we compared the expression of Slc30a3 and Cd62, two genes that are anti-correlated at the subtype level based on scRNAseq19. Slc17a7 and Gad1 are expressed in excitatory and inhibitory neurons, respectively. They are thus almost never expressed in the same neuron in the cortex. To quantify the mutual exclusivity of Slc17a7 and Gad1 in neurons, we defined the exclusivity index $E = \frac{P(\text{Gad1} | \text{Slc17a7}) P(\text{Gad1})}{P(\text{Gad1} | \text{Slc17a7}) P(\text{Gad1})}$, where $P(\text{Gad1} | \text{Slc17a7})$ indicates the probability of a cell expressing at least ten counts of Gad1 conditioned on the least counts of Slc17a7, and $P(\text{Gad1})$ indicates the probability of a cell expressing at least ten counts of Gad1 in all filtered neurons.

BARseq2 recapitulated the mutual exclusivity between these two genes (Fig. 2j), but a small number of neurons did express both Slc17a7 and Gad1 (gray cells in Fig. 2j). This could be caused by overlapping cells (that is, an inhibitory neuron and an excitatory neuron at the same $x/y$ position, but in different $z$ planes were merged together in the maximum projection images) or cell segmentation errors (two adjacent cells incorrectly segmented as a single cell). Because the sections we used were 10-µm thick, comparable to the diameter of an average neuron, the latter source of error was likely to be more common. To test this type of error was similar to doublets in droplet-based scRNA-seq techniques. Assuming that the mutual exclusions of Slc17a7 and Gad1 were absolute, then we could estimate the ‘doublet’ rate as the ratio between the probability of neurons expressing both genes and the product of the probabilities of neurons expressing either gene. Using this formula, we estimated the doublet rate of BARseq2 to be 7.5%, which is in a similar range as droplet-based scRNA-seq.
In addition to cells that express both Gad1 and Slc17a7 at substantial levels, most cells that expressed one of the two genes dominantly also had nonzero expression of the other gene, albeit at much lower levels. This noise floor could be caused by mRNAs in dendrites that were incorrectly assigned to other neurons. Because the expression levels of these genes in the somata were much higher than those in the dendrites, this type of error was unlikely to substantially affect the determination of excitatory and inhibitory neurons.

Similarly, consistent with a previous scRNA-seq study, BARseq2 also confirmed the observation that Slc30a3 was more highly expressed in subtypes of excitatory neurons that did not express Cdh24 compared to projection neurons that did express Cdh24 (Extended Data Fig. 5c; FDR < 0.05). We filtered out genes with low expression (average counts per million (CPM) < 100), then ranked genes primarily by the number of datasets where they were DEGs and secondarily by average fold change, and selected the top five ranked genes.

To examine if multiplexing affects detection sensitivity, we probed for Slc17a7, Slc30a3 and Gad1 either as a separate three-gene panel or as part of the 65-gene panel (20 cadherins and 45 marker genes). The mean expression densities across laminar positions for the three genes were similar between the three-gene panel and the 65-gene panel (Extended Data Fig. 3c). Overall, we observed extremely high performance of the PT and CT subtypes in all cases, while L6b was slightly better predicted in high-depth datasets. The cell-typing method always predicted IT cells correctly, but not always the correct layer (L2/3, L5, L6 and Car3; Extended Data Fig. 5g). This is consistent with the observation that IT type forms a continuum in single-cell datasets, making it difficult to fully separate subtypes by layer. Finally, the PT type proved to be the most difficult cell type to predict. While all PT cells were correctly annotated as PT (Extended Data Fig. 5h), numerous L2/3 IT and L5 IT cells were wrongly annotated as PT, in particular in high-depth datasets (Extended Data Fig. 5g). We believe that this was due to an imbalance in the marker panel, with PT markers showing higher expression than markers from other cell types. We tested various normalization procedures to overcome this effect but found that results were insensitive to normalization overall (Extended Data Fig. 5f).

Using this panel and cell-typing method, we determined the transcriptomic types of excitatory neurons in motor cortex using BARseq2 (Fig. 3b). Most transcriptomic types were found enriched in the correct layers. One exception to this was the L6 Car3 IT type. In general, few L6 Car3 IT neurons were identified by BARseq2. Furthermore, even though L6 Car3 IT neurons were predominantly in L6, some were identified in L2/3 by BARseq2 (Fig. 3c). This result was surprising, given that L6 Car3 IT neurons, when present, were only rarely mistyped as L2/3 in our preliminary analyses (Extended Data Fig. 5g). L6 Car3 IT neurons were only rarely detected in the datasets used to select markers, so we expect that using additional data will lead to more robust marker selection and better cell-typing performance with BARseq2. These optimizations, however, are beyond the scope of this paper.

Gene expression in barcoded neurons. Gene expression in Sindbis-infected barcoded neurons largely reflect the gene expression in non-barcoded neurons. For example, the expression of the excitatory marker Slc17a7 and the inhibitory marker Gad1 remained mutually exclusive in barcoded neurons in both auditory cortex and motor cortex (Extended Data Fig. 6d,c). This mutual exclusivity was preserved despite an overall reduction in mRNA expression (Extended Data Fig. 6e; median read of 38 in barcoded cells in both auditory and motor cortex, compared to 64 and 48 in non-barcoded auditory and motor cortex (Extended Data Fig. 6f,g)), respectively). Similarly, Slc30a3 remained differentially expressed across barcoded excitatory neurons with or without Cdh24 expression as it was in non-barcoded excitatory neurons (Extended Data Fig. 6f; P = 1 × 10^{-10} using rank-sum test, n = 810 neurons). Although our observations cannot rule out the possibility that a small subset of genes (for example, viral response genes) may be disrupted by Sindbis infection, these results suggest that the coexpression relationships of most genes in Sindbis-infected neurons reflect those in noninfected cells.

Analysis of BARseq2 gene expression and projection dataset. For analysis of BARseq2 datasets with both gene expression and projections, we first evaluated the mutual exclusivity of Slc17a7 and Gad1 expression (see below). For this purpose, the neurons were filtered with the same thresholds as in the gene-only dataset. For all other analyses, we used a more relaxed filtering to compensate for the reduced gene expression in barcoded cells, requiring neurons to have at least five counts of Slc17a7 or Gad1. In this filtered set, neurons were considered excitatory if the counts of Slc17a7 were larger than the counts of Gad1, and were considered inhibitory if the counts of Gad1 were larger than the counts of Slc17a7. Projection data were log normalized as in previous studies\(^5\). We further normalized the projection strengths of each area to two previous clustered BARseq datasets\(^6\) and used a random forest classifier to assign neurons to projection clusters.

To find cadherins that were differentially expressed across major projection classes and between auditory and motor cortex, we performed rank-sum tests for pairwise comparisons among major classes or the two areas for each cadherin and calculated the FDGs.

Projection modules were identified using NMF\(^3\). To find the variance in projections explained by cadherins and/or laminar positions (Extended Data Fig. 8), we used Gaussian process regression to predict projection modules using the laminar position of neurons as a predictor and linear regression to predict projection modules using the expression of individual cadherins. The variance explained by each predictor was calculated by comparing the variances of the prediction models to the SDs of the projection data. To predict the probability of projection modules from the expression of cadherins, we calculated the Spearman correlation between the coefficients for projection modules and gene counts. To generate the plots of differential gene expression in Fig. 6c, we sorted the neurons by the coefficients for projection modules and smoothed gene expression using a window of 101 neurons.

Projections of excitatory and inhibitory neurons. BARseq2 accurately observed the fact that projection neurons in the cortex are predominantly excitatory and express the excitatory marker Slc17a7, not the inhibitory marker Gad1. To distinguish between excitatory and inhibitory neurons, we categorized a neuron as excitatory or inhibitory if (1) the neuron had higher expression of the excitatory marker Slc17a7 or the inhibitory marker Gad1, respectively, and (2) the marker was expressed at greater than five reads in the cell. This threshold resulted in 2.496 excitatory neurons (947 in auditory cortex and 1,549 in motor cortex) and 240 inhibitory neurons (100 in auditory cortex and 140 in motor cortex; Fig. 6d). Consistent with previous observations, most cortical projection neurons identified by BARseq2 were excitatory (Fig. 6c). However, we also identified a small fraction of inhibitory projection neurons. Some of these neurons could be caused by ‘doublets’ as discussed above. Consistent with this hypothesis, the inhibitory projection neurons (and some excitatory projection neurons) in motor cortex expressed both Gad1 and Slc17a7 at similar levels (Extended Data Fig. 6g). However, inhibitory projection neurons in auditory cortex expressed only Gad1, not Slc17a7 (Extended Data Fig. 6h), suggesting that these were real inhibitory projection neurons. This observation was consistent with previous reports of rare inhibitory projection neurons in the cortex\(^5\). We did not further analyze these inhibitory projection neurons.

We also observed many excitatory neurons without projections (Fig. 6d,c), similar to those observed in BAseq experiments. These neurons were likely non-projecting excitatory neurons and neurons that project only locally or to neighboring cortical areas\(^7\) that we did not sample.
expressed across the same pairs of major classes in V1 and ALM as shown by scRNA-seq (Extended Data Fig. 7a). Several cadherins, including 
Cdh17 and Cdh11, were differentially expressed with the opposite signs in scRNA-seq and in BARseq (Extended Data Fig. 7b). However, these cadherins were not consistently expressed across motor and auditory cortex. For example, 
Cdh7 was expressed at a significantly higher level in PT neurons than CT neurons in motor cortex (P < 10^{-4}; Fig. 5c), but at a lower level in PT neurons than CT neurons in auditory cortex (P = 0.0011, not statistically significant at FDR < 0.05). It is thus likely that these differences between observations by BARseq and by scRNA-seq reflect area-to-area differences, not methodological differences. These results confirm the differential expression of cadherins across major classes identified by BARseq.

Projection differences across transcriptionally defined IT subtypes. BARseq confirmed known biases in projection patterns across transcriptional IT subtypes in auditory cortex (Extended Data Fig. 7c). Previous studies using both
barcoding-based strategies and single-cell tracing have identified distinctive projection patterns for two transcriptional subtypes of IT neurons, IT3 (L6 IT) and IT4 (L6 Car3 IT)5,6. To test if we could capture the same projection specificity of transcriptional subtypes, we mapped projection patterns to projection clusters identified in a previous study in auditory cortex, and used a combination of gene expression and laminar position to distinguish four transcriptional subtypes of IT neurons5. These subtypes were defined consistently with a previous study7 for ease of comparison. Specifically, we defined IT1 as neurons with depths of less than 590 µm, IT2 as neurons with depths between 590 and 830 µm and did not express Cdh13; IT3 as neurons between 590 and 830 µm that expressed Cdh13 or neurons deeper than 830 µm that expressed Slc30a3; and IT4 as neurons deeper than 830 µm that did not express Slc30a3.

As expected, the two transcriptional subtypes (IT3 and IT4) predominantly found in L5 and L6 were indeed more likely to project only to the ipsilateral cortex, without projections to the contralateral cortex or the striatum (P = 4 × 10^{-4} comparing all transcriptional subtypes with only ipsilateral cortical projectional neurons) in IT3/IT4 to the fraction of them in IT1/IT2 using Fisher’s test; Extended Data Fig. 7c). Between IT3 and IT4, IT4 neurons were more likely to project ipsilaterally (58% of IT3 neurons compared to 92% of IT4 neurons; P = 1 × 10^{-4} using Fisher’s test), whereas IT3 neurons were more likely to project contralaterally (66% of IT3 neurons compared to 44% of IT4 neurons; P = 5 × 10^{-4} using Fisher’s test). Thus, BARseq recapitulated known projection differences across transcriptional subtypes of IT neurons.

Cadherin coexpression module analysis. To extract robust modules of coexpressed cadherins, we used a previously developed approach to combine multiple datasets by meta-analysis, a crucial step to attenuate technical and biological noise33,34. Briefly, we built coexpression networks using the Spearman correlation for seven scRNA-seq datasets in the motor cortex23, accessed from the NeMO archive and subset to the following subclases: “L2/3 IT”, “L4/S1T”, “L5 IT”, “L6 IT”, “L6 IT Car3”. We ranked each network, then averaged the networks to obtain our final meta-expression network. We then applied hierarchical clustering with average linkage and extracted modules using the dynamic tree-cutting tree algorithm35.

To compute the association between coexpression modules and projection patterns, we framed the association as a classification task: can we predict projection patterns from module expression? First, we generated labels by binarizing each projection pattern—cells with a projection strictly greater than the median projection strength were marked as positives. Next, we generated predictors by computing gene module expression as the average log(CPM + 1) across all genes in the module. We reported the association strength (classification results) as an AUROC. To compute the association between coexpression modules and cell type, we used a similar approach, using clusters defined by the Brain Initiative Cell Census Network5,6 as labels. For visualization, cell types were organized according to the following procedure: cell types were reduced to a centroid by taking the median expression for each gene, then cell types were clustered according to hierarchical clustering with average linkage with correlation-based distance.

Validation of cadherin correlates of IT projections using in situ hybridization and retrograde labeling. To confirm that 
Cdh18, Cdh12 and Pcdh19 correlated with ipsilateral, contralateral and strial projections, respectively, we performed CTTB retrograde labeling from the projection targets and performed FISH against Slc17a7, Slc30a3 and the cadherins in both A1 and M1 (Extended Data Fig. 9a; see Supplementary Table 1 for injection coordinates). We then quantified cadherin expression and CTTB labeling in IT neurons that had sufficient DAPI signals and expressed both Slc17a7, an excitatory cell marker, and Slc30a3, which labeled the majority of IT neurons (Extended Data Fig. 9b). Neurons that had weak and/or ambiguous CTTB signals were excluded from the analyses. Indeed, we observed that the three cadherins were expressed at higher levels in CTB+- neurons in both areas despite notable overlap in expression between CTB+ and CTB- neurons (Extended Data Fig. 9c–e). This overlap was expected because CTB was unlikely to have labeled all neurons that projected to the areas that we sampled with BARseq. For example, in a previous study, we found that less than half of neurons with projections detected by BARseq were also labeled by injection of CTB into the same target area5. These results thus provide further support for the finding that cadherins correlate with similar projections in both A1 and M1.

Statistics and reproducibility. No statistical method was used to predetermine sample size, but our sample sizes are similar to those reported in previous publications8,9. No data were excluded from the analyses. Because only wild-type animals were used and the findings did not rely on comparison across animals, the experiments were not randomized and the investigators were not blinded to allocation of animals during experiments and outcome assessment. All statistical tests performed are indicated in the text. Two-tailed tests and Bonferroni correction were used for all P values reported unless noted otherwise. Wherever indicated, FDRs were computed according to the Benjamini–Hochberg procedure20. All statistical tests used were non-parametric except when statistical significance was estimated for the Pearson correlation (Fig. 6a). When estimating statistical significance for the Pearson correlation, normal distribution was assumed, but this was not formally tested.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw target area sequencing data (Fig. 4c; SRR12247894, SRR12245390 and SRR12245389) and scRNA-seq data (Fig. 2g–i) are deposited at the Sequence Read Archive (SRX1716225). Raw in situ sequencing images (Figs. 2–4) are deposited at the Brain Image Library (https://doi.org/10.5281/zenodo.350789) and example annotations from the dissected brain slices and other data and intermediate processed sequencing data are deposited at Mendeley Data (https://doi.org/10.17632/jxz89mv4k.2).

Code availability

Processing scripts are deposited at Mendeley Data (https://doi.org/10.17632/jxz89mv4k.2).

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Acknowledgements

The authors thank members of the MAPseq core facility; H. Zhan, Y. Li and N. Gemmill, for MAPseq data production; K. Matho and Z. J. Huang for distance coordinates in motor cortex; H. Zhan, L. Yuan, H. L. Gilbert, K. Matho, J. Kebschull and D. Fürth for useful discussions; and W. Wadolowski, B. Burbach, K. Lucere and E. Fong for technical support. This work was supported by the National Institutes of Health (5RO1NS073129, 5R01DA038093, RF1MH111432 and U01MH119013 to A.M.Z, R01MH13095 and R01LM012736 to J.G. and U19MH114821 to A.M.Z. and J.G.), the Brain Research Foundation (BFR-SIA-2014-03 to A.M.Z.), DARPA MCRONS (D16PC0008 to A.M.Z.), Paul Allen Distinguished Investigator Award (to A.M.Z.), Simons Foundation (350789 to X.C.) and ZOAR/ALLEN INST (SCVF) to A.M.Z. and Robert Lourie award (to A.M.Z.). This work was additionally supported by the Assistant Secretary of Defense for Health Affairs endorsed by the Department of Defense, through the FY18 RMRP Discovery Award Program (W81XWH1910083 to X.C.) (see references). Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. In conducting research using animals, the investigators adhered to the laws of the United States and regulations of the Department of Agriculture.

Author contributions

Y.-C.S., X.C. and A.M.Z. conceived the study. Y.-C.S. and X.C. optimized and performed BARseq. Y.-C.S., X.C. and H.Z. collected BARseq data. X.C., S.F. and J.G. analyzed data. Y.-C.S., X.C. and S.E. selected gene panels. X.C. and S.L. compared gene expression between BARseq and Allen ISH. Y.-C.S. and X.C. performed retrograde tracing combined with FISH validations. Y.-C.S., X.C., S.F. and A.M.Z. wrote the paper.
Competing interests
A.M.Z. is a founder and equity owner of Cajal Neuroscience and a member of its scientific advisory board. The remaining authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41593-021-00842-4.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41593-021-00842-4.

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Peer review information
Nature Neuroscience thanks Kenneth Harris and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | Optimization of BARseq2 for detecting endogenous mRNAs. a, Relative sensitivity (means and individual data points) of BARseq2 in detecting Slc17a7 using the indicated fixation times, normalized to that achieved with 5 mins of fixation. n = 3 for 480 mins and n = 4 for other conditions. b, Rolony counts for Slc17a7 using either random primers or specific primers at two different concentrations. The two concentrations used were 5 µM (low) and 50 µM (high) for random primers, and 0.5 µM (low) and 5 µM (high) for specific primers. Lines indicate means and dots/crosses represent individual samples. n = 2 slices for each condition. c, d, BARseq2 sensitivity compared to RNAscope. c, Spot density detected by BARseq2 or RNAscope in each 100 µm bin along the laminar axis in auditory cortex. Error bars indicate standard errors. The dashed line indicates linear fit for Slc30a3 and Cdh13. Slope = 1.65 and R² = 0.73. n = 5 slices for both BARseq2 and RNAscope. d, Shows the means and individual samples for each gene. e, f, Positions of rolonies across five sequencing cycles using the original (e) or the optimized (f) sequencing protocol. Scale bars = 10 µm. g, h, The distribution of minimum distance between rolonies imaged in the first cycle and in the fifth cycle using the original or the optimized protocol. h, The distribution of absolute rolony intensities for the first sequencing cycle (i) and relative rolony intensities after 6 sequencing cycles and one stripping step, normalized to the intensities in the first sequencing cycle (j). Amino-allyl dUTP concentrations used are indicated. In (i), n = 63,852 rolonies for 0.08 µM and n = 113,235 rolonies for 0.5 µM; in (j), n = 128,976 rolonies for 0.08 µM and n = 113,235 rolonies for 0.5 µM.
Extended Data Fig. 2 | Laminar distribution of cadherins in auditory cortex (green) and motor cortex (brown). In both cortical areas, cortical depth is normalized so that the bottom and the top of the cortex match between M1 and A1.
Extended Data Fig. 3 | Comparison between BARseq2 and Allen gene expression atlas. Gene expression patterns in auditory cortex identified by BARseq2 are plotted next to in situ hybridization images of the same genes in Allen gene expression atlas (ABA) and the quantified laminar distribution of the gene in both datasets. Only genes that had coronal images in the Allen gene expression atlas are shown. Blue lines indicate the boundaries of the cortex in both BARseq2 and ABA images. In the laminar distribution plots, dots represent values from two BARseq2 samples (purple) and one ABA sample (blue) per gene. Lines indicate means across samples.
Extended Data Fig. 4 | The distribution of read counts per cell for the indicated genes in auditory cortex (green) and motor cortex (brown). Asterisks indicate genes with significant difference in expression between the two areas (p < 0.05 using two-tailed rank sum test after Bonferroni correction). p values after Bonferroni correction are indicated on top.
Extended Data Fig. 5 | Transcriptomic typing using BARseq2. a,b, Slc30a3 expression in excitatory neurons with or without Cdh24 expression in single-cell RNAseq (a) from Tasic, et al. or in BARseq2 (b). A cell is considered expressing Cdh24 if the expression is higher than 10 RPKM in RNAseq or 1 count in BARseq2. Red crosses indicate means and green squares indicate medians. c, Expression density (means and individual data points) across laminar positions for the indicated genes. n = 3 slices for the three-gene panel and n = 5 slices for the 65-gene panel. d, Precision and recall of cell typing using the marker gene panel across nine single cell datasets. N = 9 independent datasets shown in (e). In each box, the center shows the median, the bounds of the box show the 1st and 3rd quartiles, the whiskers show the range of the data, and points further than 1.5 IQR (Inter-Quartile Range) from the box are shown as outliers. e, Breakdown of average performance for each cell type in each dataset. The datasets are: scSSALM and scSSV1 are single cell SmartSeq datasets from ALM and V1 respectively. All other datasets are BICCN M1 datasets and the name indicates the technology used (sc = single cell, sn = single nuclei, Cv2/3 = Chromium v2/3, SS = SmartSeq). f, Average cell typing performance for six normalization strategies. N = 9 independent datasets shown in (e). The box plots are generated in the same way as (d). g, Confusion matrix showing overlap between prediction and annotations, normalized by predictions. This plot emphasizes precision; it indicates the probability that a given prediction was correct. h, Confusion matrix showing overlap between prediction and annotations, normalized by annotations. This plot emphasizes recall; it indicates the probability that a given annotation was recovered.
Extended Data Fig. 6 | Correlating gene expression to projections using BARseq2. a, Relative sensitivity of BARseq2 to barcodes (solid line) and endogenous mRNAs (dashed line) using the indicated concentration of Phusion DNA polymerase. Sensitivities are normalized to the original BARseq condition (Ctrl). Circles and crosses show individual data points across n = 2 slices. b, Correlation between pairs of genes in barcoded cells (y-axis) and in non-barcoded cells (x-axis) as determined by BARseq2. Shuffled data (yellow) are also plotted for comparison. c,d, Slc17a7 (x-axes) and Gad1 (y-axes) expression in barcoded neurons in auditory (c) or motor cortex (d). Only neurons with more than 10 counts in either gene are shown. e, The distributions of read counts per barcoded neuron (solid lines) or non-barcoded neuron (dashed lines) in auditory (green) and motor (brown) cortex. f, Slc30a3 expression in barcoded excitatory neurons with or without Cdh24 expression in BARseq2. A cell is considered expressing Cdh24 if the expression is higher than 1 count. Red crosses indicate means and green squares indicate median. g,h, Slc17a7 (x-axes) and Gad1 (y-axes) expression in barcoded projection neurons in motor (g) or auditory cortex (h). Excitatory and inhibitory neurons are color-coded as indicated.
Extended Data Fig. 7 | BARseq2 reveals projection and gene expression differences across major classes and IT subtypes. 

**a.** Differential gene expression across major classes (IT, PT, and CT) observed using BARseq2 and single-cell RNAseq. Each dot shows the difference in mean expression of a gene across a pair of major classes observed using BARseq2 (y-axis) or single-cell RNAseq (x-axis). Differences in expression that were statistically significant (FDR < 0.05 using two-tailed rank sum tests) in both A1 and M1 as shown by BARseq2 are labeled purple; otherwise they are labeled yellow. The single-cell RNAseq data used were collected in the visual cortex and anterior-lateral motor cortex. 

**b.** The fraction of ITi-Ctx neurons in four transcriptomic types of IT neurons in auditory cortex. ITi-Ctx neurons have only ipsilateral cortical projections and no striatal projections or contralateral projections. The number of ITi-Ctx neurons and neurons with other projection patterns for each transcriptomic type are labeled on top of the pie charts.

**c.** The projection strengths for contralateral (y-axis) and ipsilateral (x-axis) cortical projections for each IT neuron in auditory cortex. IT1/IT2 neurons are labeled blue and IT3/IT4 neurons are labeled red.
Extended Data Fig. 8 | Variance in projections explained by cadherins and laminar positions. Box plots of variance in each projection modules explained by the indicated predictors after 100 iterations of 10-fold cross validation. Boxes indicate second and third quartiles and whiskers indicate minimum and maximum values excluding outliers. Outliers are shown in red.
Extended Data Fig. 9 | Validation of correlation between cadherins and IT projections. a, Representative images of in situ hybridization in A1 (top) and M1 (bottom) slices with CTB labeling in the caudal striatum. Three marker genes and CTB labeling are shown in the indicated colors. Scale bars = 100 µm. Arrows and arrowheads indicate example CTB + and CTB- neurons, respectively. Experiments for each combination of targeted gene and CTB labeling condition (Cdh12 with contralateral labeling, Cdh8 with ipsilateral labeling, and Pcdh19 with striatal labeling) were performed in slices from two animals. b, Crops of the indicated individual channels of example neurons from (a). Scale bars = 10 µm. c–e, Cumulative probability distribution of the expression of Cdh12 (c), Cdh8 (d), and Pcdh19 (e) in neurons with or without retrograde labeling of contralateral (c), ipsilateral (d), or caudal striatal (e) projections. p values from two-tailed rank sum tests after Bonferroni correction and numbers of neurons used for each experiment are indicated. N = 2 animals for each experiment.
Extended Data Fig. 10 | Cadherin co-expression modules correlate with IT projections. a, Correlation among cadherins in IT neurons in motor cortex identified in the indicated single-cell RNA-seq datasets\(^3,23\). The datasets included are: tasic_alm and tasic_v1 are single cell SmartSeq datasets from ALM and V1 respectively\(^3\); all other datasets are BICCN M1 datasets\(^23\); the name indicates the technology used (sc = single cell, sn = single nuclei, Cv2/3 = Chromium v2/3, SS = SmartSeq). b, Modularity (EGAD AUROC) of co-expression modules in BARseq2 M1 against null distribution of modularity (node permutation). BARseq2 modularity is shown by the blue lines with the corresponding p-values. P values are calculated using a one-sided non-parametric node permutation test without multiple comparison correction. c, Association (AUROC) between cadherin co-expression modules and the indicated projections. Significant associations are marked by asterisks (* FDR < 0.1, ** FDR < 0.05). d, Fractions of neurons with the indicated projections as a function of co-expression module expression. e, Distribution of associations of the indicated projection modules with gene expression. Association with significant gene module is shown by a blue line; association with single genes from that module is shown by orange lines; association with all other genes is shown by a gray density. f, Association of the three co-expression modules in transcriptomic IT neurons in the indicated datasets (AUROC, significance shown as in e).
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Software and code

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| Data collection | micro-manager (Version 1.4.23, Build 20190321) and Zeiss Zen 2012 SPS FP2 (Version 14.0.0.0) were used to capture microscopy data. |
| Data analysis   | Custom MATLAB codes are provided at Mendeley Data (http://dx.doi.org/10.17632/jnx89bmv4s.1) as indicated in the data availability section in Methods. The codes were run on MATLAB 2018b. The gene expression module analysis uses the R library “dynamicTreeCut” (v1.63.1) ran in R v4.0.0. |

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Target area sequencing data (Fig. 4) are deposited at SRA (SRR12247894, SRR12245390, and SRR12245389). Single-cell RNAseq data are deposited at SRA (SRR13716225). Raw in situ sequencing images (Fig. 2-4) are deposited at Brain Image Library (https://download.brainimagelibrary.org/06/35/0635a0b3b0954c7e/). Other data and processed sequencing data are deposited at Mendeley Data (http://dx.doi.org/10.17632/jnx89bmv4s.1).
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Sample size

No sample-size calculation was performed. Because this manuscript focuses on reporting the development of the BARseq2 technique, we performed BARseq2 on three biological replicates to ensure the reproducibility of the technique.

Data exclusions

No data was excluded

Replication

The main BARseq2 experiments were performed on three animals and multiple findings from BARseq2 are validated using other techniques. The main new findings were validated using FISH and retrograde labeling. This validation experiments was replicated successfully across two animals for each experiment.

Randomization

Randomization was not applicable because the findings did not rely on comparison across different biological samples, but among different neurons. The scoring of neuronal properties (i.e. gene expression and projections) was automated and did not involve human scoring.

Blinding

Blinding was not relevant to the main dataset because the findings did not rely on comparison across different biological samples, but among different neurons. The scoring of neuronal properties (i.e. gene expression and projections) was automated and did not involve human scoring. Other experiments (i.e. comparison across experimental conditions) were also quantified without human input.

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Laboratory animals

Male C57BL/6J mice of age 8-10 wks were used. The animals were housed at maximum of 5 in a cage on a 12 hrs on/12 hrs off light cycle. The temperature in the facility was kept at 22 °C with a range not exceeding 20.5 °C to 26 °C. Humidity was maintained at around 45-55% not exceeding a range of 30-70%. A list of animals used is provided in Supp. Table S1.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected in the field.

Ethics oversight

All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee protocol 19-16-10-07-03-00-4 at Cold Spring Harbor Laboratory.

Note that full information on the approval of the study protocol must also be provided in the manuscript.