**NEUROGENOMIC IMAGING**

Conservation and divergence of cortical cell organization in human and mouse revealed by MERFISH

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The human cerebral cortex has tremendous cellular diversity. How different cell types are organized in the human cortex and how cellular organization varies across species remain unclear. In this study, we performed spatially resolved single-cell profiling of 4000 genes using multiplexed error-robust fluorescence in situ hybridization (MERFISH), identified more than 100 transcriptionally distinct cell populations, and generated a molecularly defined and spatially resolved cell atlas of the human middle and superior temporal gyrus. We further explored cell-cell interactions arising from soma contact or proximity in a cell type–specific manner. Comparison of the human and mouse cortices showed conservation in the laminar organization of cells and differences in somatic interactions across species. Our data revealed human-specific cell-cell proximity patterns and a markedly increased enrichment for interactions between neurons and non-neuronal cells in the human cortex.

The human cerebral cortex comprises billions of cells of distinct types (1). The spatial organizations and interactions of these cells play a critical role in shaping and maintaining various brain functions (2). For instance, interactions between neuronal and non-neuronal cells are essential for axonal conduction, synaptic transmission, and tissue homeostasis and are required for normal functioning of the brain (3, 4). Disruption of such cell-cell interactions contributes to various neurological disorders, such as autism (5), schizophrenia (6), and Alzheimer’s disease (7). Yet we have only a limited understanding of the organizations and interactions of different cell types in the human cortex.

Recent single-cell RNA sequencing (scRNA-seq) analysis has revealed a diversity of transcriptionally distinct cell populations in the human middle temporal gyrus (MTG) (8). Efforts that combine scRNA-seq with microdissection (8), and more recently in situ sequencing to target 120 genes (9), have revealed the laminar organization of these transcriptionally defined neuronal cell types—in particular, the excitatory neurons—in the human MTG. These studies, however, did not elucidate the spatial relationship between cell types at high resolution, and a systematic characterization of cell-cell interactions among this high diversity of cell types is still lacking. Single-cell transcriptomics and epigenomics analyses have also provided rich insights into the evolution of cellular diversity and molecular signatures of cell types in the cortices of mice, marmosets, and humans (8, 10, 11), but how the spatial relationship and interactions between different cell types vary across species remains largely unclear.

**Single-cell transcriptome imaging of the human cortex**

Single-cell transcriptome imaging allows in situ gene expression profiling of individual cells and, hence, high-resolution spatial mapping of cell type organization in complex tissues. Here we describe single-cell transcriptome imaging of the human brain performed with multiplexed error-robust fluorescence in situ hybridization (MERFISH) (12). We carried out MERFISH measurements of the human MTG and superior temporal gyrus (STG) from fresh-frozen neurosurgical and postmortem brain samples, targeting 4000 genes (Fig. 1A). These genes included 764 differentially expressed marker genes in cell clusters derived from single-nucleus SMART-seq data of the MTG (8) and additional expressed genes that were largely randomly selected to increase the gene coverage. This allowed us to include potential marker genes not identified in the SMART-seq data, as well as functionally important genes such as ligands and receptors. To overcome the high autofluorescence background in human tissues due to lipofuscin, we photobleached the samples with light-emitting diode arrays (13) before MERFISH imaging. We then used expansion microscopy (14) to reduce the molecular crowding associated with imaging a large number of genes (15, 16).

Individual RNA molecules were identified and assigned to segmented cells to determine the single-cell expression profiles (Fig. 1B and fig. S1). We imaged five tissue sections from neurosurgical MTG samples (from two male individuals, 36 and 32 years old) and five sections from postmortem STG samples (from two male individuals, 29 and 42 years old). MERFISH expression data showed excellent reproducibility between replicates (fig. S2, A and B); high correlation between neurosurgical MTG and postmortem STG samples, albeit with a lower total transcript count in the latter, likely due to RNA degradation (fig. S2C); and high correlation with bulk RNA sequencing data (fig. S3).

To test whether the molecular crowding associated with imaging 4000 genes caused substantial reduction in the detection efficiency, we performed MERFISH imaging on 250 of the 764 marker genes (in two expanded tissue sections for detection efficiency assessment and in three additional unexpanded sections to increase the number of cells imaged). The detection efficiency of the 4000-gene measurements was, on average, ~57% of that of the 250-gene measurements on expanded sections, with high correlation between the two measurements (fig. S4).

**Cell type classification of the human cortex**

We used single-cell expression profiles derived from the 4000-gene MERFISH data to identify transcriptionally distinct cell populations. First-level clustering detected excitatory and inhibitory neurons, as well as major subclasses of non-neuronal cells such as microglia, astrocytes, oligodendrocytes, oligodendrocyte progenitor cells (OPCs), endothelial cells, and mural cells, as characterized by the marker genes identified by SMART-seq (8) (fig. S5).

We then performed separate clustering analyses of inhibitory and excitatory neurons from the MERFISH data, which corresponded closely with those independently determined from the SMART-seq data (fig. S6). To combine information from both datasets, we performed integrated analysis of MERFISH and SMART-seq data (fig. S7, A and B). This analysis classified inhibitory neurons into four subclasses (denoted by marker genes SST, VIP, PVALB, and LAMP5, respectively) and excitatory neurons into nine subclasses (L2/3 IT, L4/5 IT, L5 IT, L6 IT, L6 IT CAR3, L5 ET, L5/6 NP, L6 CT, and L6b), with most subclasses further subdivided into multiple clusters (Fig. 1C). Because non-neuronal cells were depleted from the SMART-seq dataset (8), we identified clusters within individual subclasses of non-neuronal cells from the 4000-gene MERFISH data alone (Fig. 1C and fig. S7C). Altogether, we identified a total of 125 transcriptionally distinct cell populations in the human MTG and STG—29 excitatory, 39 inhibitory, and 75 non-neuronal clusters (Fig. 1C and fig. S7)—revealing not only a high diversity of neurons but also a high diversity of non-neuronal cells in the human cortex. To include the 250-gene data for downstream analysis, we performed supervised classification to predict their cell type labels (at the cluster level for neurons and the subclass level for non-neuronal cells) on the basis of annotations from the 4000-gene data.

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Quantitative analysis of the cell composition using the MERFISH data showed that the human MTG and STG (white matter excluded) were composed of 26% excitatory neurons, 11% inhibitory neurons, and 63% non-neuronal cells (Fig. 1D). The excitatory neurons were predominantly intratelencephalic (IT) neurons (~93%), with only a small fraction of non-IT neurons (L6 CT, L5 ET, L5/6 NP, and L6b cells) (Fig. 1E, left). The IT neurons were subdivided into 46% L2/3 IT, 18% L4/5 IT, 19% L5 IT, 13% L6 IT, and 4% L6 IT CAR3 cells (Fig. 1E, middle). The inhibitory neurons were composed of 13% LAMPS, 26% PVALB, 30% SST, and 31% VIP cells (Fig. 1E, right).

Next, we compared cell composition in human and mouse cortices. The human STG contains the auditory cortex, whereas the human MTG does not have a counterpart in mice, with the mouse temporal association area (TEa) considered to be the closest ortholog. We thus considered two MERFISH datasets that cover several regions of the mouse cortex: (i) our recently reported 258-gene MERFISH dataset of the primary motor cortex (MOp) (17) and (ii) a dataset generated from additional MERFISH experiments on a more posterior part of the mouse cortex, containing the visual cortex (VIS), auditory cortex (AUD), and TEa (Fig. S8) using a similar gene panel and experimental protocol as for the MOp (17). Similar cell compositions were observed across these different mouse cortical regions (Fig. 1, D and E).

However, the cell composition of the human MTG and STG was substantially different from that of the mouse cortical regions. We observed a lower proportion of excitatory neurons and a higher proportion of glial cells (including astrocytes, oligodendrocytes, OPCs, and microglia) in human MTG and STG and four mouse cortical regions (MOp, VIS, AUD, and TEa).
times the ratio observed in mice (Fig. 1D) (17, 20). The excitatory-to-inhibitory neuron ratio was 2.3 in humans, in line with recent independent measurements (9, 11) and one-third of the ratio observed in mice (Fig. 1D) (17, 20).

Among the excitatory neurons, the non-IT neuron proportion dropped from 29% in mice to 7% in humans (Fig. 1E, left), consistent with recent observations that L5 ET and L6 CT are less abundant in primates than in mice (11). The dominance of IT neurons in humans suggests an increased emphasis of intracortical communications. For inhibitory neurons, we observed a decrease in the proportion of PVALB neurons and an increase in the proportion of VIP neurons in humans relative to mice (Fig. 1E, right). In behaving animals, VIP interneurons regulate inhibition of excitatory neurons through inhibition of other interneurons, and such disinhibition facilitates modulation of sensory response and network dynamics by behavioral state and learning (21). The observed increase in VIP interneuron proportion thus suggests a potential mechanism for the enhanced capability of state-dependent sensory processing and learning-related neuronal dynamics in humans.

**Spatial organizations of cells in the human and mouse cortices**

In situ identification of cell types by MERFISH allowed us to map their spatial organizations. In humans, we observed a laminar organization of IT neurons across the cortical depth, whereas other excitatory neurons (including L5 ET, L5/6 NP, L6 CT, and L6b) were populated mostly in the deep layers (Fig. 2 and figs. S9 and S10), as expected (8, 9, 22). Among inhibitory neurons, VIP and LAMP5 were enriched in the upper layers (L1 to L3), whereas PVALB and SST were more broadly distributed across the layers (Fig. 2 and figs. S9 and S10), consistent with previous observations (8, 9, 22). At the cluster level, inhibitory neurons also adopted a laminar organization, with many inhibitory clusters primarily restricted to one cortical layer or even a subportion of a layer (Fig. 2B, middle, and fig. S9), enriching and refining the knowledge of layer-restricted inhibitory neuron distributions (8). These spatial organizations of neurons were largely similar to those observed in the mouse cortex (figs. S10 and S11) (17).

Despite the overall conservation of laminar organization, we also found differences between humans and mice for some neuronal cell types. For instance, the L6b neurons were broadly dispersed in L6 and extended into L5 and white matter in the human MTG and STG, whereas in mice L6b formed a thin layer at the bottom of L6 (Fig. 3A), consistent with

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**Fig. 2. Laminar organization of cell types in the human and mouse cortices.** (A) Spatial maps of subclasses of excitatory neurons, inhibitory neurons, and glial cells determined by MERFISH in a human MTG slice and a mouse slice containing VIS, AUD, and TEa. Indicated subclasses are shown in colors; other cells are in gray. (B) Cortical-depth distribution of excitatory (top), inhibitory (middle), and non-neuronal (bottom) clusters in the human MTG. The dashed lines mark the approximate layer boundaries. WM, white matter.
previous findings (22, 23). The L4/5 IT neurons formed a dense and thin layer in the human MTG and STG, giving rise to a substantially higher density of excitatory neurons in L4 (Fig. 3, B and C, top). By contrast, the density of excitatory neurons in the mouse cortex was more uniform across L2/3 to L6 (Fig. 3C). L4 is known to vary between different cortical regions, so whether this difference is region- or species-specific remains an open question, although the several mouse cortical regions that we examined exhibited a similar density profile. We also observed a different cortical-depth dependence for the excitatory-to-inhibitory neuron ratio between humans and mice (Fig. 3C).

Non-neuronal cells also exhibited laminar organization in the human cortex. Oligodendrocytes were enriched in the deeper layers and white matter and depleted in the upper layers (L1 to L3) (fig. S10) (9). Although astrocytes, microglia, OPCs, endothelial cells, and mural cells were dispersed across all cortical layers at the subclass level (fig. S10), these cell types exhibited laminar organization at the cluster level (Fig. 2B, bottom). For example, the ASC i cluster was localized in L1, likely representing interlaminar astrocytes (24); ASC ii was enriched in L1 and L2/3; ASC iii and ASC iv were enriched in L2/3 and L4; ASC v to ix were dispersed across L2 to L6; and ASC x and xi were enriched in L6 and white matter (Fig. 2B, bottom), improving our understanding of astrocyte diversity and organization (8, 25, 26).

Cell-cell interactions in the human and mouse cortices

High-resolution MERFISH measurements of the spatial relationship between cells allowed us to predict cell-cell interactions arising from somatic contact or paracrine signaling (27, 28), which can be inferred from soma contact or proximity that occurred at a higher frequency than random chance. Our MERFISH images showed frequent somatic contact or proximity between cells (Fig. 4A and fig. S12, A and B). Although the cell density in the human cortex was one-third of that in mice (fig. S12A), was observed between two subclasses of cells. We then determined whether this frequency was significantly greater than random chance, thus reflecting an enrichment, by comparing the observed frequency with the expected frequencies from random spatial permutations that disrupted the spatial relationship between neighboring cells while preserving the local density of each cell type (figs. S13 to S15).

We observed cell type–specific patterns for soma contact or proximity enrichment in the expanded human cortex. To examine whether these potential cell-cell interactions were cell type specific, we considered cell types at the subclass level and calculated the frequency at which soma contact or proximity, determined on the basis of centroid distance (fig. S12A), was observed between two subclasses of cells. We then determined whether this frequency was significantly greater than random chance, thus reflecting an enrichment, by comparing the observed frequency with the expected frequencies from random spatial permutations that disrupted the spatial relationship between neighboring cells while preserving the local density of each cell type (figs. S13 to S15).
to form contacting or proximity pairs among cells within the same subclass (Fig. 4B, left, and fig. S17). These results were further supported by examining the distances from individual neurons to their nearest neighbors in the same or different types (Fig. 4C, top). This tendency was also observed in mice but to a lesser degree for some neuronal types (Fig. 4, B and C), consistent with the previous observation that inhibitory neurons in mice tend to form intrasubtype nearest-neighbor pairs (29). Some non-neuronal cell types also exhibited such tendency for intratype soma proximity, but with noticeable differences between humans and mice. For example, we observed enrichment for soma contact or proximity among astrocytes in humans but not in mice (Fig. 4B and fig. S17). It has been shown that the processes of neighboring astrocytes intermingle substantially more in humans than in mice (25, 26, 30). Whether these observations are related to our findings here remains an open question.

A notable difference between humans and mice was observed for glial-vascular interactions. The human, but not mouse, cortex exhibited enrichment for soma contact or proximity between glial and vascular cells (Fig. 4B and fig. S17). MERFISH images showed that the cell bodies of oligodendrocytes and microglia were often clustered around vascular structures formed by endothelial and mural cells (Fig. 5A). These observations are corroborated by a recent electron microscopy study (30), which showed that oligodendrocyte and microglial cell bodies are adjacent to blood vessels, whereas astrocytes contact blood vessels primarily with their end feet but not cell bodies. Quantifications of MERFISH images showed that more microglia and oligodendrocytes, but not astrocytes, formed somatic contacts with blood vessels in humans than in mice (Fig. 5B and fig. S18).

Cross-species differences in cell-cell interactions were also observed between neurons and glial cells—in particular, oligodendrocytes and microglia. We observed substantial enrichment for soma contact or proximity between neurons and oligodendrocytes, including both mature oligodendrocytes and OPCs, in humans (Fig. 4B and fig. S17). Although somatic contacts between neurons and oligodendrocytes were also observed in mice and could represent bona fide interactions, the frequency of such events did not significantly exceed that expected from random chance. Moreover, a single neuron often formed contacts with several oligodendrocytes and OPCs in humans, whereas such
multiway contacts were not enriched above random chance in mice (Fig. 5, A and C).

In humans, among OPCs, a specific subpopulation exhibited a higher tendency to contact neurons. Our analyses of both MERFISH and SMART-seq data (8) showed that ~50% of the OPCs expressed glutamate decarboxylase 1 (GAD1), a gene encoding an enzyme that synthesizes γ-aminobutyric acid (GABA), whereas glutamate decarboxylase 2 (GAD2) and the GABA transporter gene VGAT (SLC32A1) were not expressed in OPCs (fig. S19A). Compared with GAD1-negative OPCs, GAD1-positive OPCs contacted neurons at a higher frequency (Fig. 5, A19, B, and C).

Finally, our data revealed differences in microglia-neuron interactions in humans and mice. In the human MTG and STG, microglia were frequently juxtaposed with neurons (Fig. 5A), likely representing satellite microglia (31). In addition, these satellite microglia exhibited a greater degree of enrichment for soma contact with or proximity to excitatory neurons as opposed to inhibitory neurons (Fig. 4B, left; fig. S17; and Fig. 5, A and D). Moreover, among excitatory IT neurons, the tendency to contact microglia decreased with cortical depth (Fig. 5D). By contrast, no significant enrichment in microglia-neuron contact was observed in the mouse cortex (Fig. 4B, fig. S17, and Fig. 5, A and D). Furthermore, we identified ligand-receptor pairs enriched in contacting microglia and IT neurons (Fig. 5E, fig. S17, and Fig. 5D).

Discussion
In this study, we demonstrated 4000-gene MERFISH imaging of human brain tissues. Our MERFISH images enabled in situ identification of >100 neuronal and non-neuronal cell populations and comprehensive mapping of the spatial organization of these cells in the human MTG and STG, resulting in a molecularly defined and spatially resolved cell atlas with high granularity. The cell composition in these human cortical regions differed markedly from that observed in several mouse cortical regions. The spatial organization of cells showed both common and divergent features between humans and mice.
Although we cannot exclude the possibility that some of these differences are due to different cortical regions, we consider this less likely because the different mouse cortical regions that we assessed exhibited similar cell type compositions and organizations, and the same was true for the human MTG and STG.

These high–spatial resolution cell atlases allowed us to systematically characterize proximity-based somatic interactions in a cell type–specific manner and revealed differences in cell-cell interactions between humans and mice. The differences were particularly pronounced for interactions between neuronal and non-neuronal cells. We observed substantially increased enrichment for soma contact or proximity between neurons and oligodendrocytes in the human cortex compared with the mouse cortex. Perineuronal oligodendrocytes (32) can provide metabolic support to neurons (36). Hence, the observed increase in contact enrichment between oligodendrocytes and neurons may be a result of evolutionary adaptation to higher energy demands during the firing of individual neurons in the human brain (37). In addition, we observed preferential enrichment for contact or proximity between microglia and excitatory neurons, compared with inhibitory neurons, in the human cortex, whereas the mouse cortex did not exhibit significant enrichment for such microglia-neuron contact. Satellite microglia can help maintain tissue homeostasis (38), and human genetics evidence suggests that microglia play a protective role that lowers the incidence of some neurodegenerative diseases (39). Our observation may thus represent a functional interaction between microglia and excitatory neurons in humans. Some ligand-receptor pairs genetically associated with neurodegenerative diseases were enriched in contacting microglia-neuron pairs as compared with non-interacting microglia and neurons, suggesting a possible molecular basis underlying the observed microglia-neuron interactions and a potential connection of these cell-cell interactions to neurodegenerative diseases. Evolution of non-neuronal cells has been suggested to follow a more complex pattern than simply increasing the cell abundance; it also involves the diversification of glial cells (40). Our observations of the enhanced enrichment for interactions between neurons and glia in the human cortex further expand on this view.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1 to S20
Tables S1 to S9
References (44–54)
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