

# THE RNAscope™ MULTIPLEX *IN SITU* HYBRIDIZATION TECHNOLOGY ENABLES THE INCORPORATION OF SPATIAL MAPPING AND CONFIRMATION OF GENE SIGNATURES INTO SINGLE CELL RNA SEQUENCING WORKFLOWS

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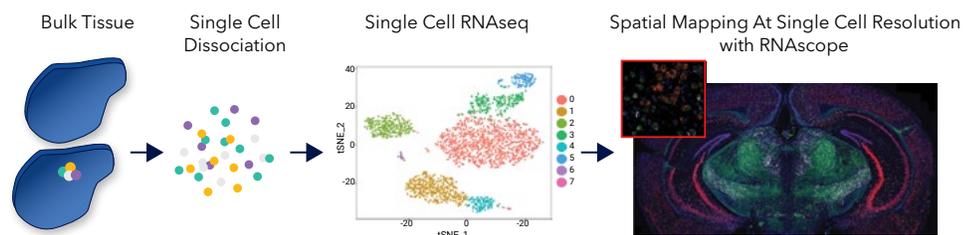
## HIGHLIGHTS

Complex, highly heterogeneous tissues, such as the brain, are comprised of multiple cell types and states. However, interrogation of these complex tissue types requires a highly sensitive, specific, and multiplexed spatial approach with single cell resolution. With the RNAscope technology, researchers can:

- Visualize the cellular heterogeneity of complex organs with the multiplexing and spatial capabilities of the RNAscope Multiplex Fluorescent and HiPlex assays
- Detect up to 12 RNA targets simultaneously in the tissue context with the RNAscope HiPlex assay
- Spatially map scRNA-seq gene profiles at the single cell level in the tissue context
- Localize newly identified cell subtypes and cell markers
- Confirm scRNA-seq results from publicly available datasets in the tissue context
- Combine the RNAscope Multiplex Fluorescent Assays with IHC/IF to simultaneously detect RNA and protein

Characterizing the transcriptomic profiles of individual cells by single-cell RNA sequencing (scRNA-seq) has become a universal tool to identify both known and novel cell populations and to understand tissue structure and function, ushering in a new era of single cell biology. This has proven to be especially true in complex organs with high cellular heterogeneity, such as the mammalian brain. However, scRNA-seq utilizes dissociated cells and results in the loss of spatial organization of the cell population being analyzed. Confirmation and spatial mapping of scRNA-seq results can be obtained using assays that retain spatial organization, such as RNA *in situ* hybridization (ISH).

The RNAscope technology is an advanced *in situ* hybridization assay that allows for the visualization of single-cell gene expression targeting RNA sequences directly in tissues. The proprietary double Z probe design in combination with the advanced signal amplification enables highly specific and sensitive detection of target RNAs in fresh frozen, fixed frozen, and formalin-fixed paraffin-embedded (FFPE) cells and tissues, with each dot representing a single RNA transcript. Therefore, this robust signal-to-noise technology allows for the detection of gene transcripts at single-molecule level with single-cell resolution. The multiplexing capabilities of the RNAscope Multiplex Fluorescent assay, with simultaneous detection of up to 4 targets, and the new RNAscope HiPlex assay, with simultaneous detection of up to 12 targets in fresh frozen tissue section, provide pivotal single cell imaging data to confirm and spatially map gene profiles identified by scRNA-seq in complex tissues<sup>1</sup> (FIGURE 1).



**FIGURE 1.** Incorporation of spatial mapping into single cell RNA sequencing workflows with the RNAscope technology.

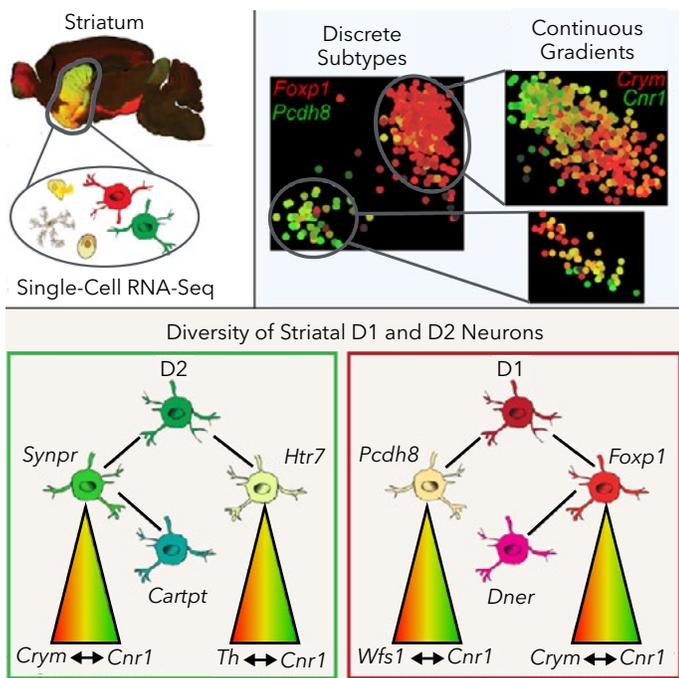


FIGURE 2. scRNA-seq reveals discrete striatal medium spiny neuronal (MSN) subtypes. Schematic depicting the striatal neuronal subpopulations identified by scRNA-seq. Images from Gokce et al., Cell Rep. 2016. 16(4): 1126-1137.

In this report, the diverse cell types in the mouse striatum that have been previously identified by scRNA-seq<sup>2</sup> (FIGURE 2) were confirmed and spatially mapped using the RNAscope Multiplex Fluorescent assay and the RNAscope HiPlex assay. The major and minor gene signatures identified by scRNA-seq, including discrete D1 and D2 medium spiny neuron (MSN) subtypes, were confirmed. Further cellular heterogeneity within the MSN subpopulations was marked by a transcriptional gradient, which was spatially resolved with the RNAscope technology. Lastly, heterogeneity within non-neuronal striatal cell types was confirmed, including vascular smooth muscle cells, endothelial cells, microglia, macrophages, and oligodendrocytes.

## RESULTS

To confirm the gene signatures identified by scRNA-seq, the two distinct D1 and D2 MSN cell types with their major and minor populations were detected in the mouse striatum using the RNAscope Multiplex Fluorescent assay. The RNAscope results confirmed scRNA-seq data showing high expression of the major D1 subtype (marked by *Drd1a*+/*Foxp1*+) and low expression of the minor D1 subtype (marked by *Drd1a*+/*Pcdh8*+) in the mouse striatum (FIGURE 3). By using 4-plex probe panels with the RNAscope Multiplex assay, these populations were further refined to visualize the D1 major, D1 minor, D2 major, and D2 minor subtypes, revealing a similar expression profile as identified by scRNA-seq (FIGURES 4-5). These expression patterns were detected across varying regions of the striatum. For example, the *Drd1a*+/*Meis2*+ cells were identified in the dorsal striatum, while the *Drd1a*+/*Dner*+ cells were located more in the ventral striatum.

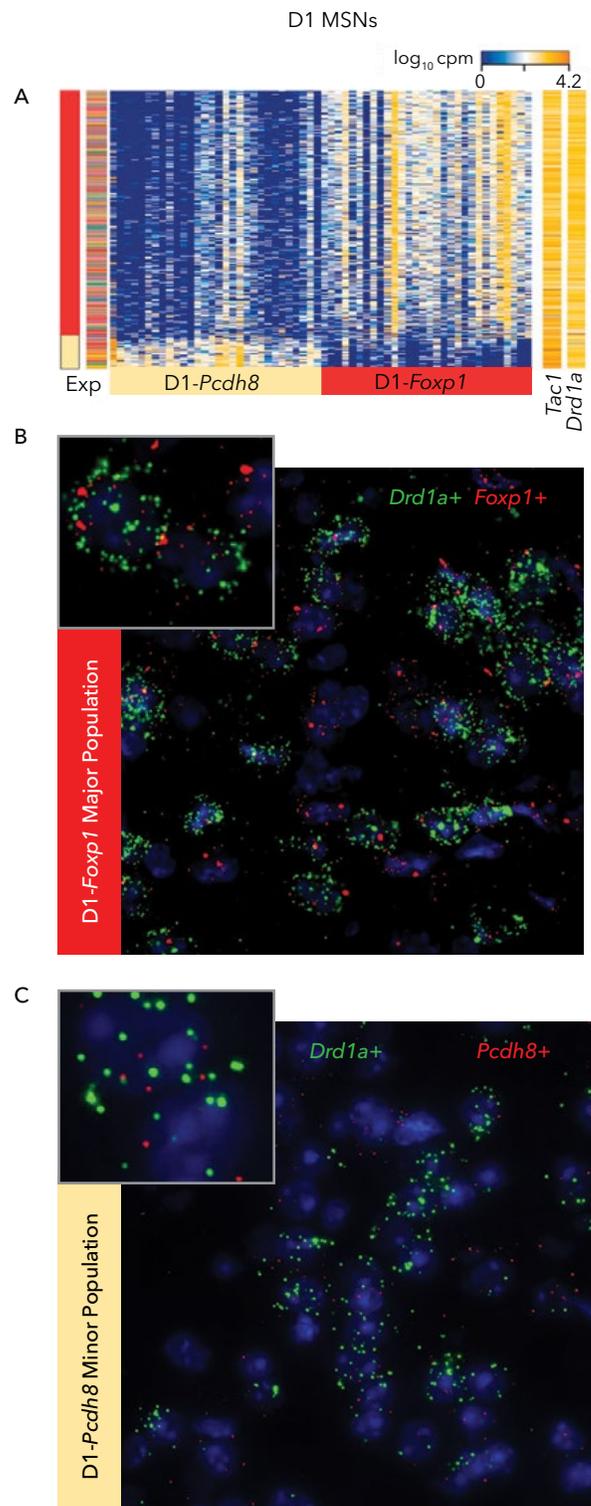


FIGURE 3. Discrete striatal medium spiny neurons (MSNs) visualized with the RNAscope Multiplex Fluorescent assay. (A) scRNA-seq identified Major and Minor subtypes of *Drd1a* MSNs, which were confirmed by the RNAscope Multiplex Fluorescent assay (B-C) to express major (*Drd1a*+/*Foxp1*+) or minor (*Drd1a*+/*Pcdh8*+) gene signatures. (A) Images from Gokce et al., Cell Rep. 2016. 16(4): 1126-1137.

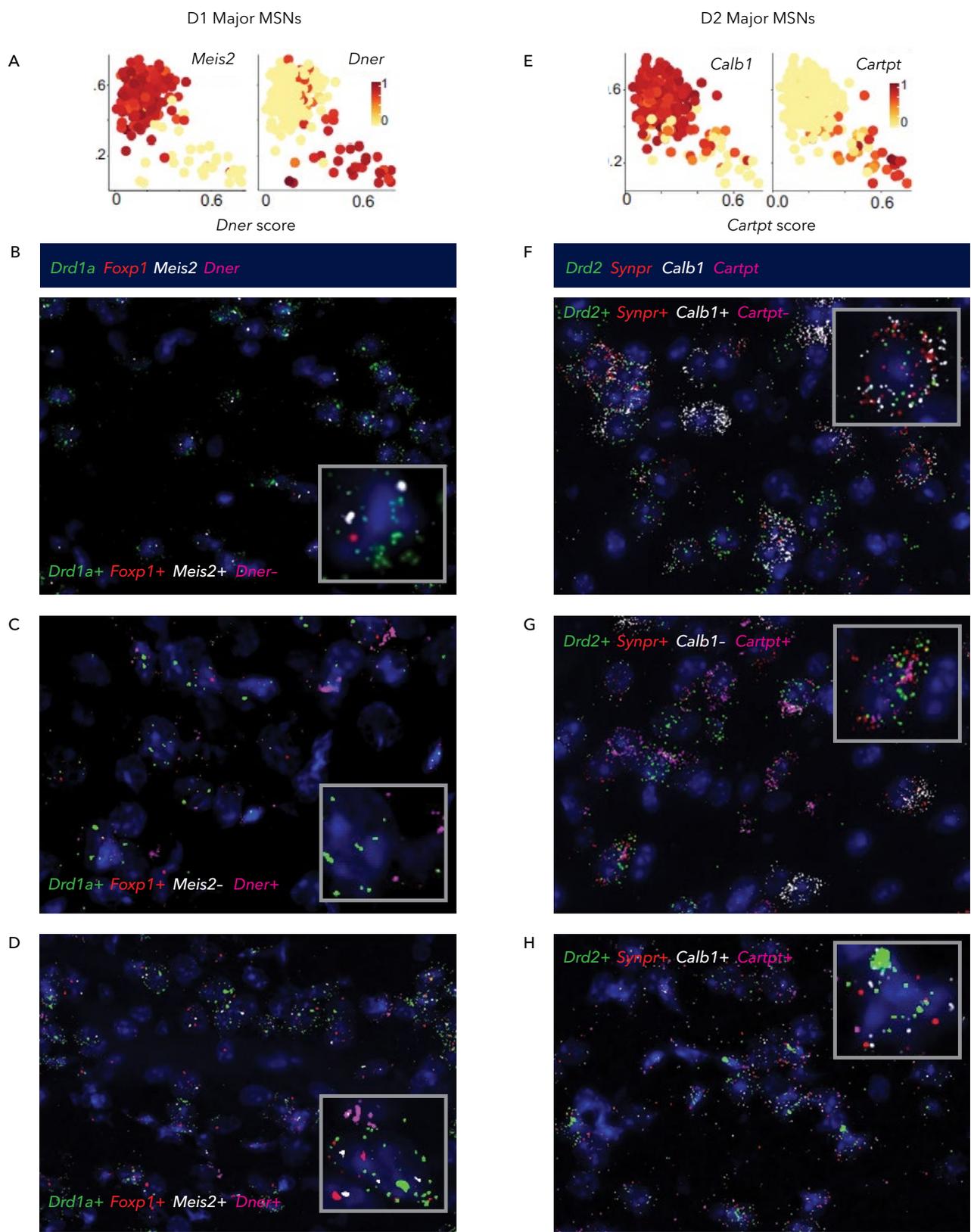


FIGURE 4. Refinement of the major MSN subtypes in the mouse striatum. (A-D) Major *Drd1a* MSNs identified by scRNA-seq (A) and confirmed by the RNAscope Multiplex Fluorescent assay (B-D) to express *Drd1a* and *Foxp1* in combination with *Meis2* and/or *Dner*. (E-H) Major *Drd2* MSNs identified by scRNA-seq (E) and confirmed by the RNAscope Multiplex Fluorescent assay (F-H) to express *Drd2* and *Synpr* in combination with *Calb1* and/or *Cartpt*. (A, E) Images from Gokce *et al.*, Cell Rep. 2016. 16(4): 1126-1137.

Interestingly, the scRNA-seq analysis by Gokce *et al.* revealed further heterogeneity in these subtypes through a transcriptional gradient<sup>2</sup>. The RNAscope Multiplex assay revealed that this

gradient had a distinctive spatial gene expression pattern, with a region that was high in *Crym* neighboring a region that was high

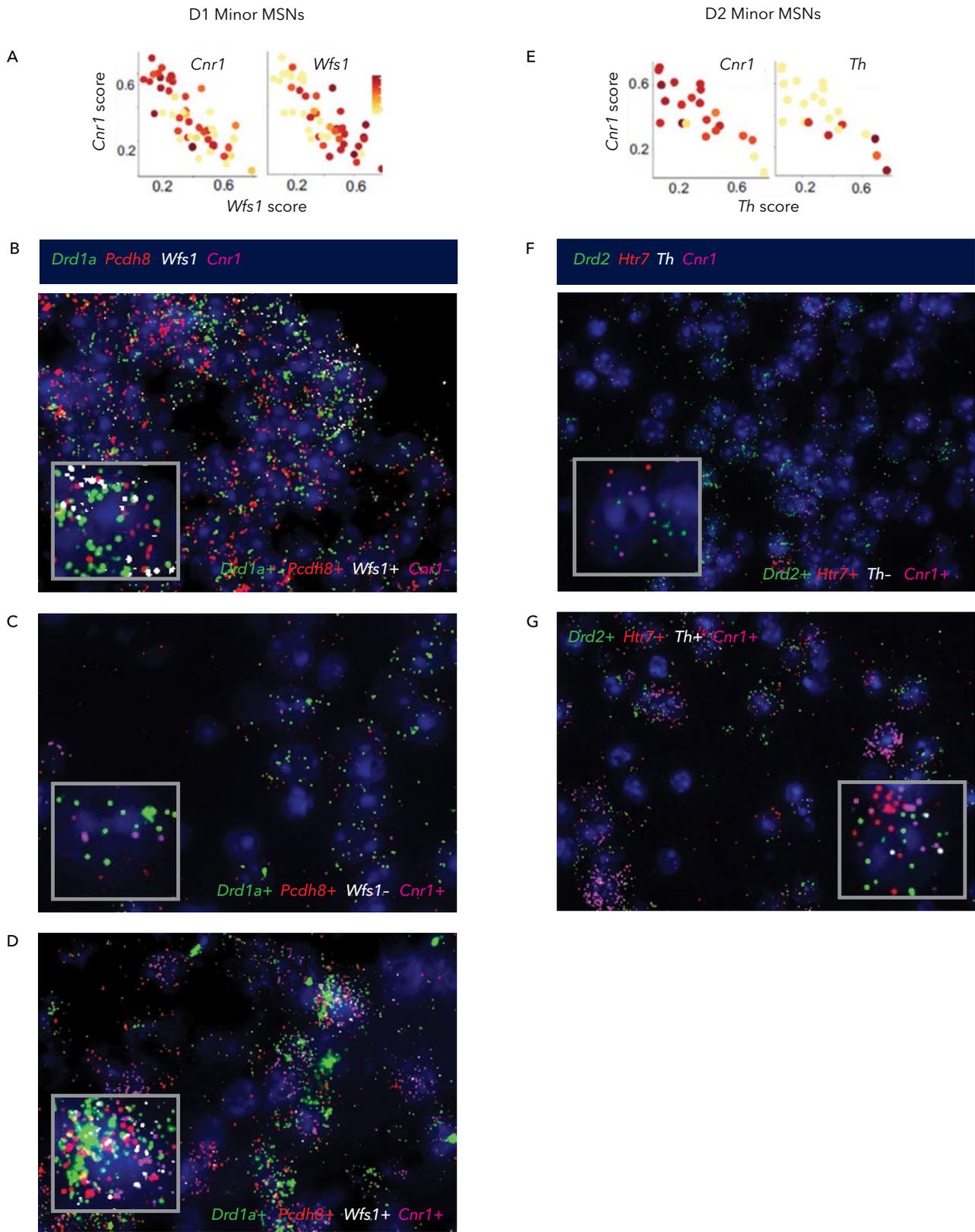
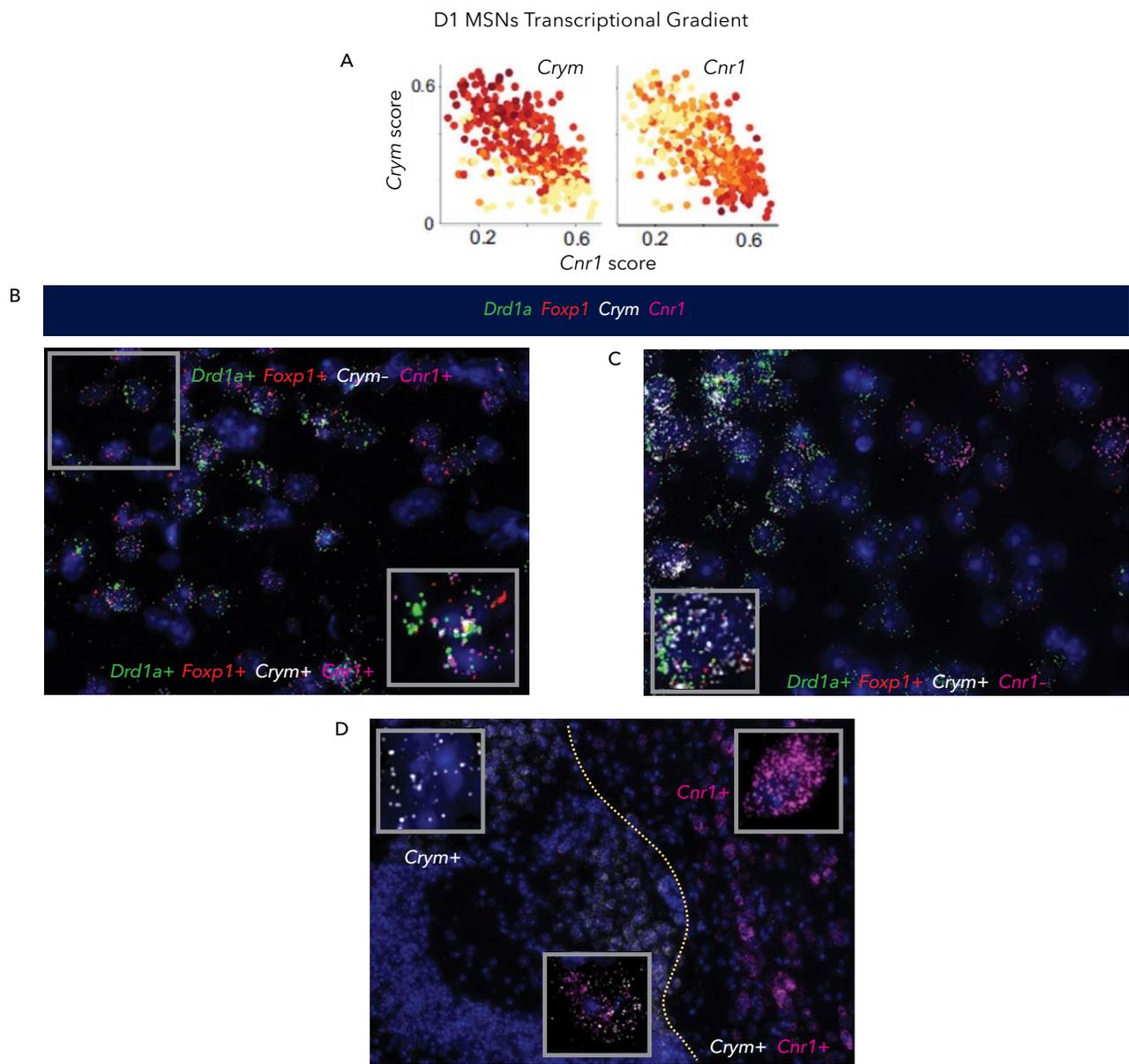


FIGURE 5. Refinement of the minor MSN subtypes in the mouse striatum. (A-D) Minor Drd1a MSNs identified by scRNA-seq (A) and confirmed by the RNAscope Multiplex Fluorescent assay (B-D) to express *Drd1a* and *Pcdh8* in combination with *Wfs1* and/or *Cnr1*. (E-G) Minor Drd2 MSNs identified by scRNA-seq (E) and confirmed by the RNAscope Multiplex Fluorescent assay (F-G) to express *Drd2* and *Htr7* in combination with *Cnr1* and/or *Th*. (A, E) Images from Gokce et al., Cell Rep. 2016. 16(4): 1126-1137.

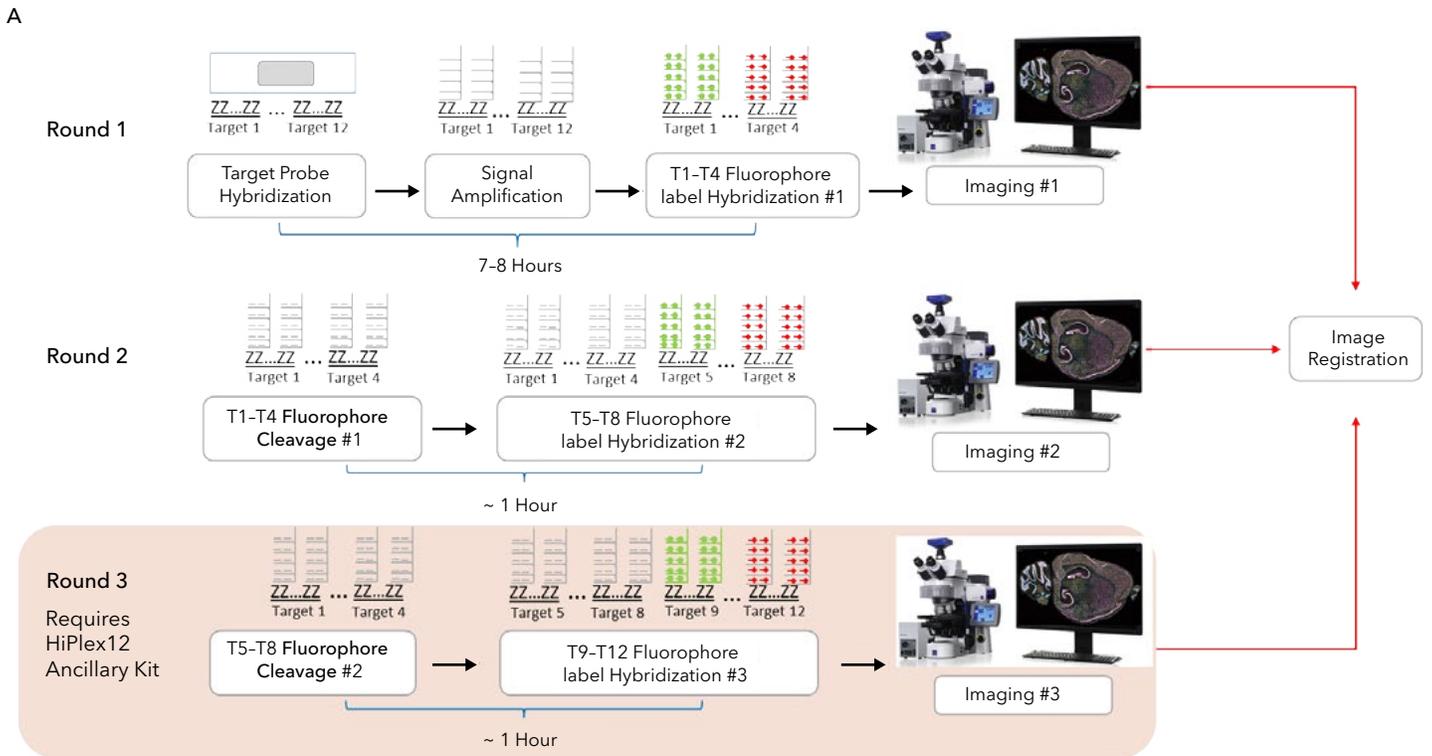


**FIGURE 6. Continuous transcriptional gradient in striatal MSNs.** (A-D) Transcriptional gradient was identified in *Drd1a* MSNs by scRNA-seq (A) and confirmed by the RNAscope Multiplex Fluorescent assay (B-D). *Drd1a*+/*Foxp1*+ cells express *Crym* and/or *Cnr1* (B, C). A distinct spatial pattern was identified for cells expressing only *Crym*, only *Cnr1*, or co-expressing *Crym1*/*Cnr1* (D). (A) Images from Gokce et al., Cell Rep. 2016. 16(4): 1126-1137.

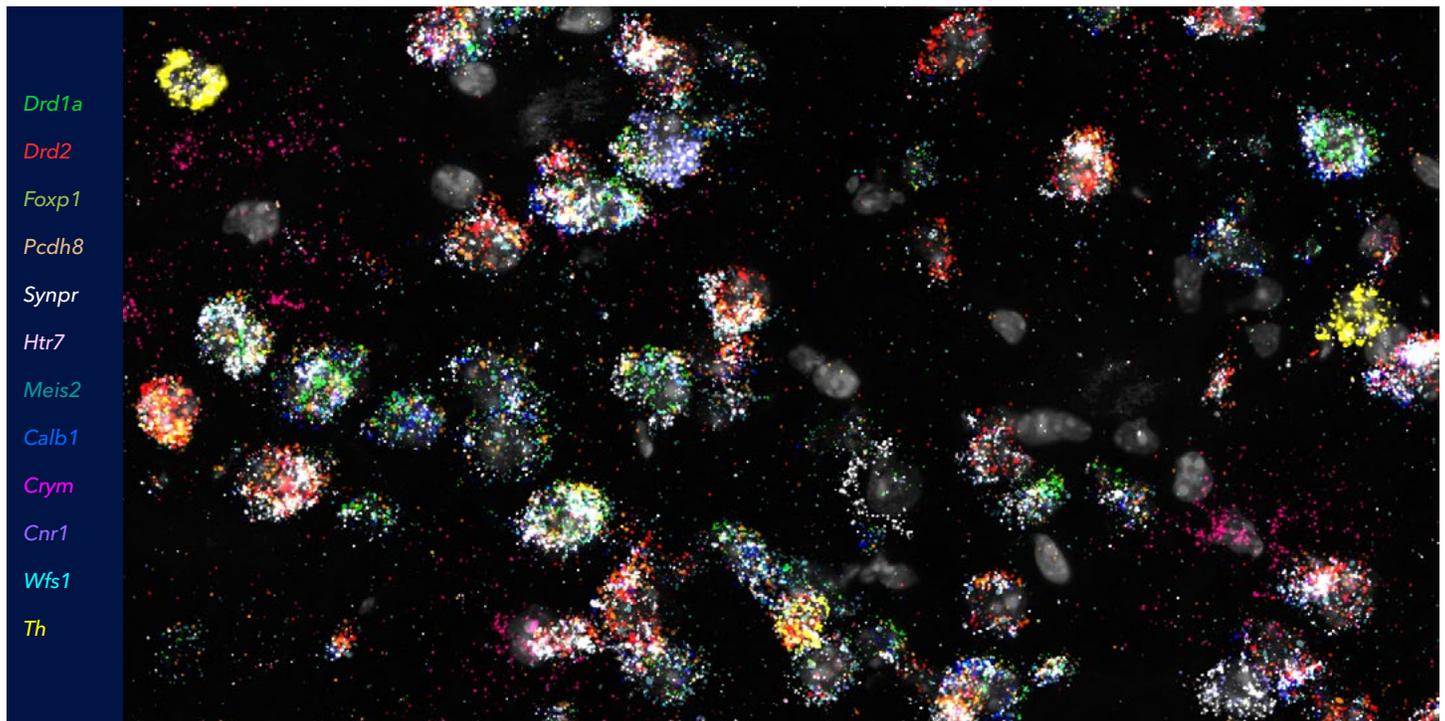
in *Cnr1*, and a common region containing cells that co-expressed *Crym* and *Cnr1* (FIGURE 6).

While multiplexing with 4 probes was enough to visualize the individual D1 and D2 subtypes on separate sections, simultaneous detection of both the D1 and D2 subtypes on the same striatal section was needed to fully assess the two populations. Therefore, the RNAscope HiPlex assay was employed, which provides signal amplification for up to 12 RNA targets on the same tissue section by performing iterative fluorescent imaging in groups

of 4 targets at a time (FIGURE 7A). Performing the RNAscope HiPlex assay with 12 targets first allowed simultaneous detection of all the D1 and D2 subtypes on the same section (FIGURE 7B). Furthermore, the RNAscope HiPlex assay can be combined with immunofluorescence (IF) to simultaneously detect RNA and protein. Using an antibody against the neuronal marker NeuN in combination with the 12 targets that characterize the D1 and D2 subtypes allowed for the visualization of these cell types amongst all of the NeuN+ neurons in the mouse striatum (FIGURE 8).



**B** RNAscope HiPlex imaging of *Drd1a* and *Drd2* MSN subtypes



**FIGURE 7.** The RNAscope HiPlex assay for 12 targets provides comprehensive spatial mapping of the D1/D2 striatal MSN subtypes simultaneously in the tissue context. (A) Experimental workflow for the RNAscope HiPlex assay. (B) Visualizing both the D1 and D2 striatal MSNs in the same mouse brain section using the RNAscope HiPlex assay for 12 targets.

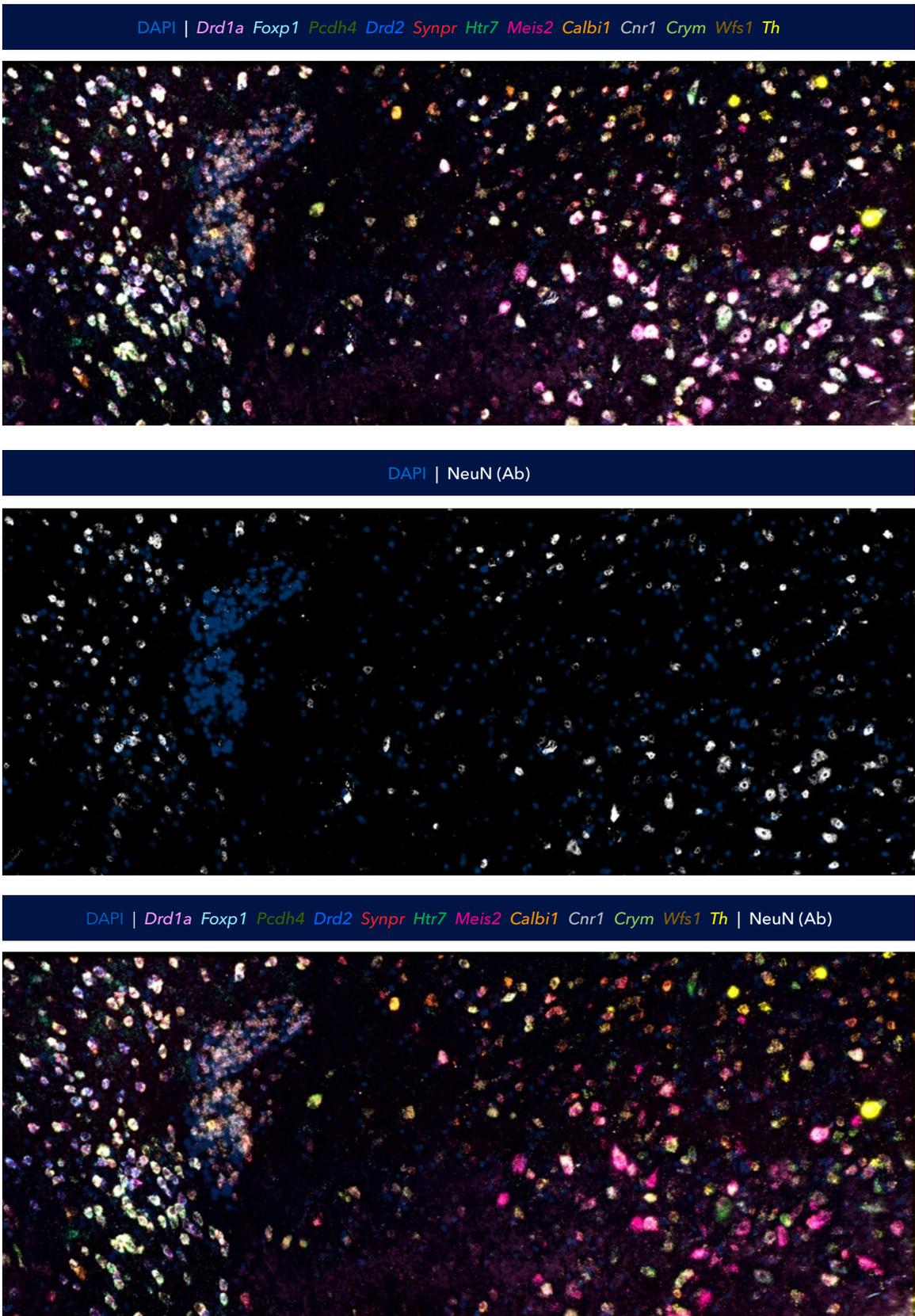


FIGURE 8. The RNAscope HiPlex assay was combined with immunofluorescence for the neuronal marker NeuN to identify the D1 and D2 gene signatures among all striatal neurons.

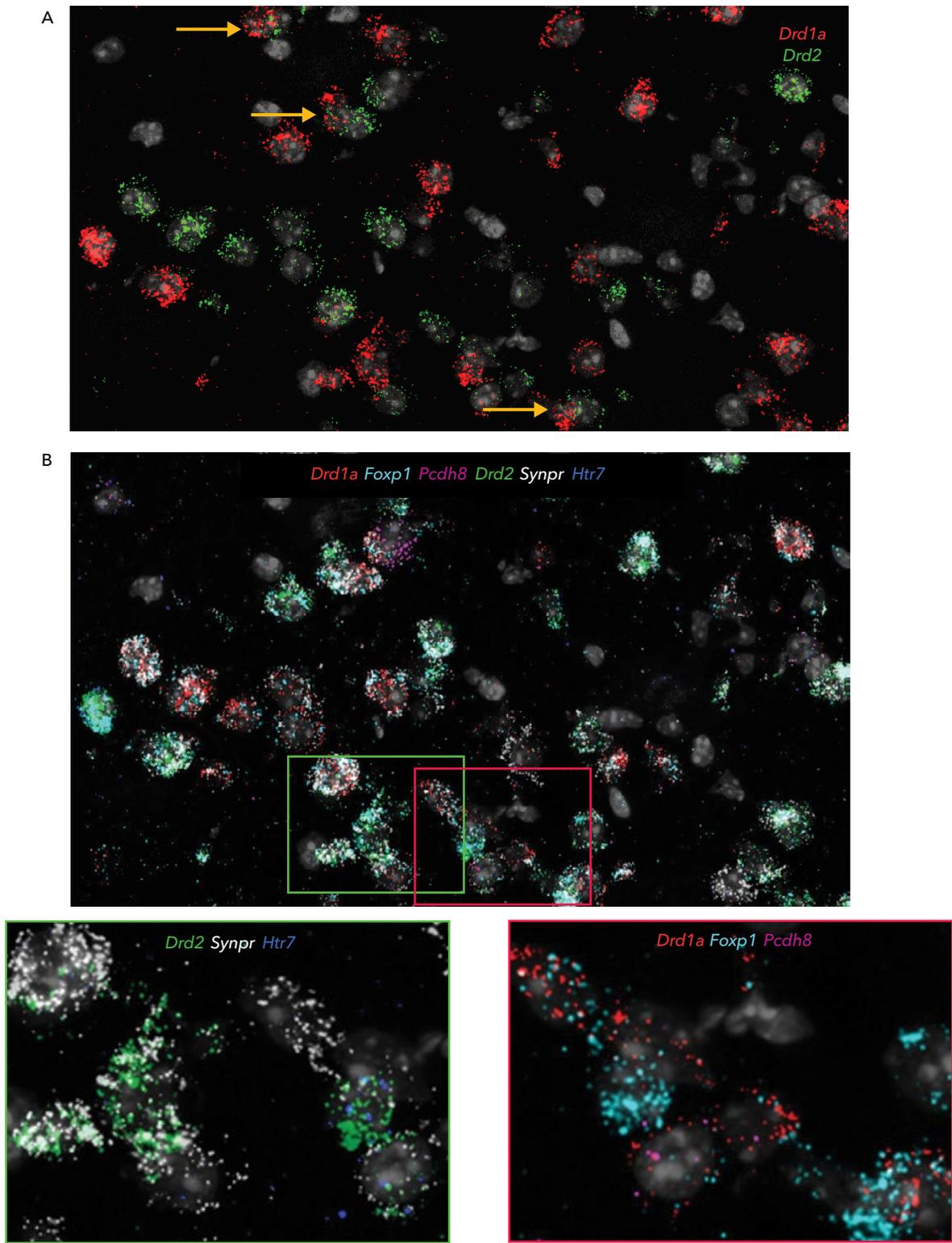


FIGURE 9. Detection of the major and minor D1 and D2 subtypes simultaneously in the tissue context with the RNAscope HiPlex assay. (A) Detection of cells co-expressing *Drd1a* and *Drd2* (marked by yellow arrows). (B) Visualization of the D1 (right) and D2 (left) major and minor populations on the same sagittal section of mouse brain.

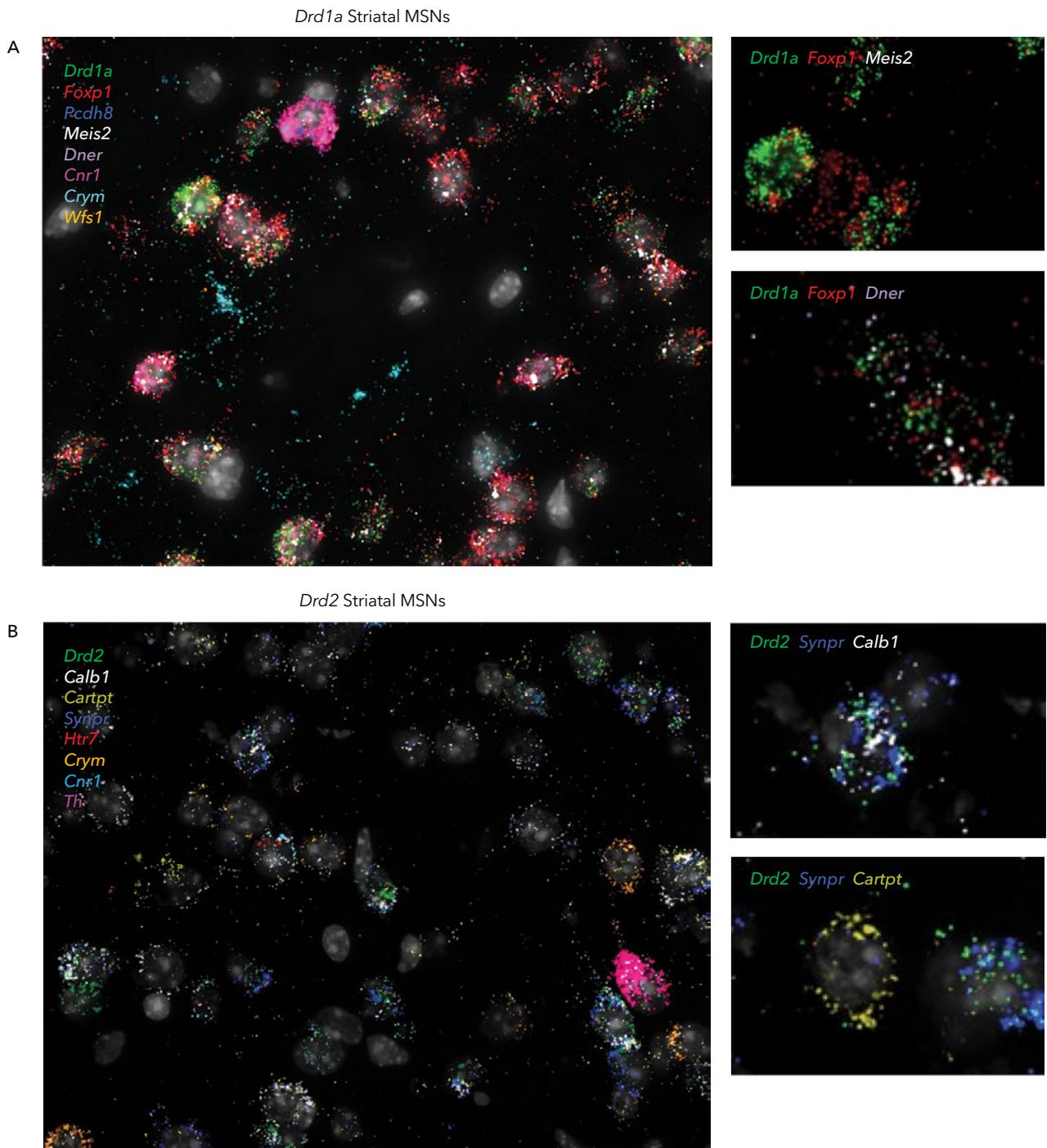


FIGURE 10. Simultaneous visualization and characterization of the D1 and D2 subtypes in the mouse striatum with the RNAscope HiPlex assay. The D1 subtypes are characterized by *Drd1a*, *Foxp1*, *Pcdh8*, *Meis2*, *Dner*, *Cnr1*, *Crym*, and *Wfs1* (A), whereas the D2 subtypes are characterized by *Drd2*, *Calb1*, *Cartpt*, *Synpr*, *Htr7*, *Crym*, *Cnr1*, and *Th* (B). Insets show cells expressing markers indicative of the major D1 (A) and D2 (B) subtypes.

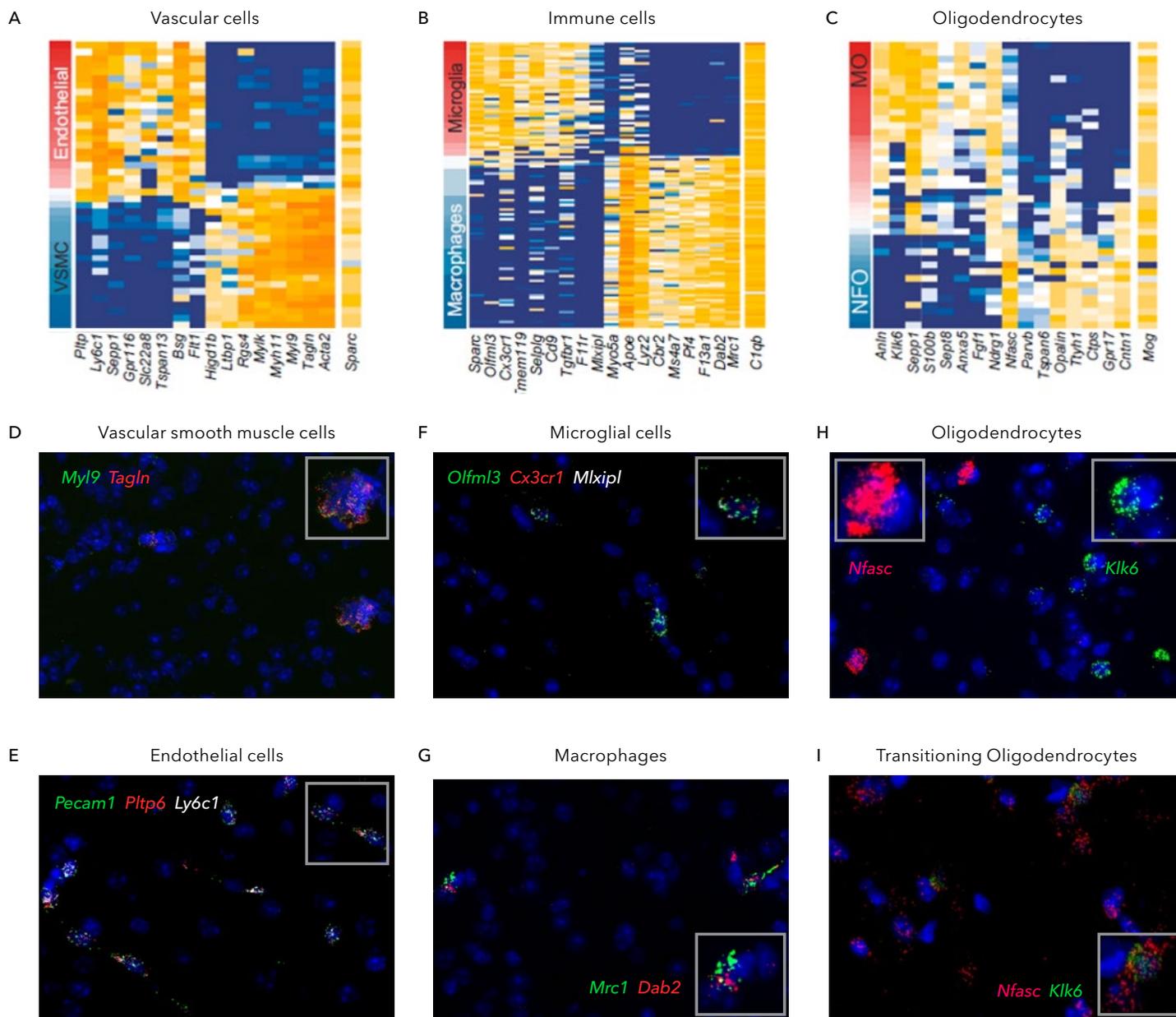


FIGURE 11. Characterization of non-neuronal striatal cell populations. scRNA-seq of the mouse striatum also identified non-neuronal cells which were confirmed by the RNAscope Multiplex Fluorescent assay. (D-E) Identification of vascular cells, including smooth muscle and endothelial cells. (F-G) Identification of immune cells, including microglia and macrophages. (H-I) Identification of oligodendrocytes (OLs), including mature OLs (marked by *Klk6*), newly forming OLs (marked by *Nfasc*), and transitioning OLs (marked by co-expression of *Nfasc* and *Klk6*). (A-C) Images from Gokce et al., Cell Rep. 2016. 16(4): 1126-1137.

Using the RNAscope HiPlex image registration software, varying probe combinations were selected from the 12-plex panel to visualize individual cell subtypes on the same section. For example, the registration software allowed for detection and confirmation of a group of MSNs that co-express *Drd1a* and *Drd2* as reported by Gokce *et al.*<sup>2</sup> (FIGURE 9A). Additionally, the D1 and D2 major and minor groups were visualized by selecting for their respective gene signatures (FIGURE 9B). The RNAscope HiPlex assay with 8 targets allowed for complete visualization of the D1 major and minor subtypes (FIGURE 10A) and D2 major and minor subtypes (FIGURE 10B) on the same section.

In addition to neuronal cells, Gokce *et al.* also identified several non-neuronal cell populations in the striatum using scRNA-seq<sup>2</sup> (FIGURE 11A-C). The RNAscope Multiplex Fluorescent assay also confirmed the presence of vascular cells and immune cells (FIGURE 11D-G). Interestingly, scRNA-seq profiling suggested the presence of 3 types of oligodendrocytes (OLs), which were confirmed with the RNAscope Multiplex Fluorescent assay: mature OLs, newly forming OLs, and then a small population of transitioning OLs that are in transition from newly forming to mature OLs (FIGURE 11H-I).

## CONCLUSION

Complex tissues with high cellular heterogeneity require single cell technologies both at the transcriptomic and spatial level to fully interrogate these cell types. This report demonstrates the capabilities of a multiplexed *in situ* transcriptomic approach for the confirmation and spatial mapping of scRNA-seq results in the highly complex and heterogeneous mouse striatum using both the RNAscope Multiplex Fluorescent and HiPlex assays. Numerous researchers from around the world<sup>3-11</sup> have utilized the RNAscope technology to incorporate spatial analyses into their scRNA-seq workflows and further single cell research in neuroscience and beyond. Single cell transcriptomics combined with spatial mapping by RNA ISH holds great promise in resolving heterogeneous tissues at cellular resolution and providing insights into cellular organization and function of diverse cell types in healthy and disease states.

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