Imaging and analysis of mouse midbrain tissue labeled by RNAscope

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We use this protocol and it's working

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ABSTRACT

Below we describe imaging, analysis and cell counts of coronal sections of mouse substantia nigra and ventral
tegmental area labeled with Slc17a6 (VGLUT2), Slc32a1 (VGAT), and Slc18a2 (VMAT2).

ATTACHMENTS

rnascope imaging and counting protocol.docx

MATERIALS

Equipment/Software	Supplier and Catalog Number
Confocal Microscope	Zeiss (LSM 880 Indimo, Axio Observer)
Plan-apochromat 20X objective, 0.8/M27	Zeiss (420650-9901-000)
Zen Black Software (v2)	Zeiss

Keywords: rnascope, counting, exhaustive counting, confocal, colocalization , mouse brain, fluorescence

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A. Imaging

1 Remove labeled slides from storage. If slides are not clean, spray both sides with a 70% ethanol 30% distilled water solution and wipe clean with lens paper.

2 Turn on computer, confocal microscope, and lasers (helium-neon, diode-pumped solid-state, argon, and diode). Start ZEN Black.

3 Place slide in holder and locate area of interest with a 5x or 10x objective. Then switch to 20X objective and focus image.

4 For general acquisition settings, adjust bit depth and pixel averaging values to ensure clear detection of puncta. We used a bit depth of 12 and pixel averaging of 8.

5 For each detection channel, set detection wavelength, gain, power, and pinhole diameter (measured in airy units [AU]) to ensure clear detection of puncta. Without compromising signal quality, choose the lowest power values possible to reduce photobleaching. Optimize detection settings to maximize signal capture while preventing bleed-through and minimizing background. For our experiment, we used the following settings:

5.1 Indigo (DAPI): Diode laser at 405nm, detection at 415-479nm, 0.5% power, 611 gain, 1.12 AU

5.2 Blue (C1): Argon laser at 488nm, detection at 500-550 nm, 0.5% power, 558 gain, 1.96 AU

5.3 Green (C2): Diode-pumped solid-state laser at 561nm, detection at 576-627nm, 0.5% power, 750 gain, 0.83AU.

5.4 Red (C3): Helium-neon laser at 633nm, detection settings not applicable, 0.5% power, 700 gain, 0.77AU.

6 If capturing a brain region larger than the 20x field of view, create an ROI using the tile settings.

7 Under the autofocus strategy section, select the Fluorescence Autofocus Strategy option. If an ROI composed of several tiles was created in step 6, set the focus strategy to every 1 tile. Note that this adds about 4-5 minutes per tile to run time.

8 Select “Run” and wait for image to be captured.

9 Save image as a CZI file.

10 Repeat steps until all sections have been imaged.

B. Image processing

11 Open desired CZI file with Zen software.

12 If tiled images were captured, use the stitching function under the processing tab. Adjust stitching settings so that no tiling artifacts are present.

13 Save stitched image as a new CZI file.

14 With the new file open, select and display only one RNAscope signal channel.

15 In the histogram section of the display tab, set gamma value to 0.45.

16 Adjust black value to minimize signal more than 5 µm away from nuclei without suppressing signal within 5 µm of nuclei.

17 Adjust white value until signal is as bright as possible without it saturating.

17.1 Continue readjusting white and black values until both criteria are optimized. Once set, do not change values for the remainder of the counting procedure.

18 Repeat steps 14-17 until all channels are processed.

19 For DAPI signal, set gamma to 0.45. Adjust the black and white values as needed during the counting procedure, e.g., to facilitate detection of weakly labeled nuclei or distinguish between closely packed nuclei.

C. Classification criteria

20 The nucleus must be circular, at least 5 µm in diameter, and the DAPI signal must be present throughout the nucleus (i.e. exclude nuclei devoid of signal in the center) (Figure 1A for positive example, Figure 2 for positive and negative examples).

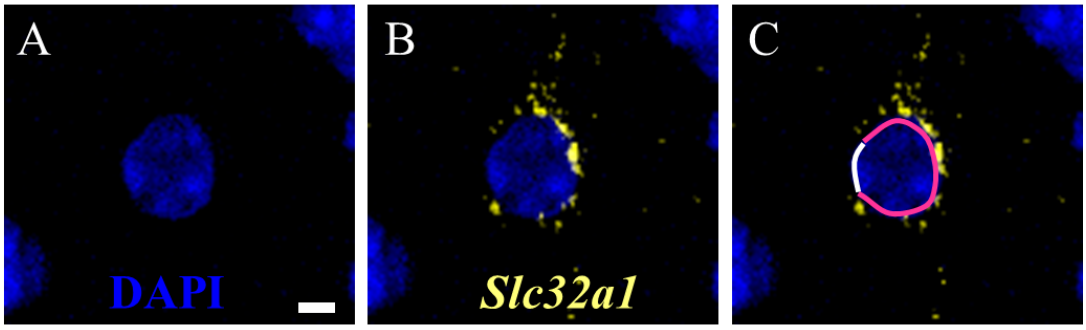

Figure 1: Positive classification of cell for RNA expression. A. Standalone nucleus stained with DAPI. This nucleus meets criteria defined in step 1 of part C. **B**. The same nucleus with RNA channel shown. C. Magenta outline shows part of nucleus perimeter surrounded by RNA signal, where white outline is where the perimeter is devoid of RNA signal. Because more than 4 puncta localize around more than 50% of the nucleus, this cell is considered positive for RNA expression. Scale bar is 5 µm.

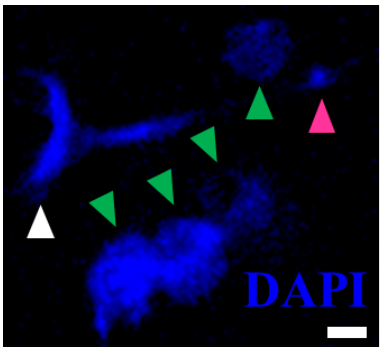

Figure 2. Examples of acceptable and unacceptable nuclei. Green arrowheads point to nuclei meeting criteria. White arrowhead points to DAPI signal rejected as nucleus due to incomplete circular morphology. Magenta arrowhead points to DAPI signal rejected for being under 5 µm in diameter. Scale bar is 5 µm

21 If a nucleus **is not** touching other nuclei, there must be at least 5 RNA puncta distributed concentrically around 50% of the nucleus and within ~2 µm of nucleus edge (Figure 1A-C).

22 If a nucleus **is** touching other nuclei, the same rules as step 2 apply except 30% of the RNA signal must unambiguously be from the cell in question (i.e. signal cannot reasonably be ascribed to a neighboring cell). Take special care to not count the same puncta twice for neighboring nuclei. (Figure 3).

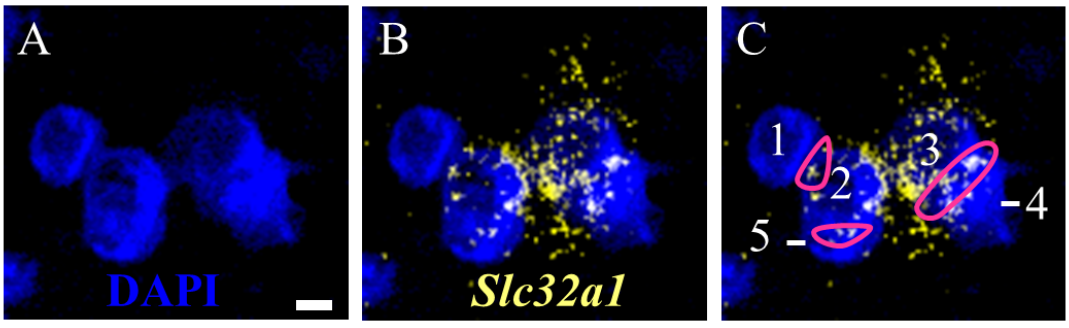

Figure 3. Classification conditions when nuclei touch.
A. DAPI channel. **B**. DAPI channel with RNA expression. **C**. Magenta outlines denote RNA signal shared by neighboring nuclei and therefore cannot be exclusively assigned to any nucleus.
Cells number 2 and 3 are considered positive for RNA expression due to sufficient RNA puncta not included in magenta outline. Cells 1, 4, and 5 do not have more than 30% of their diameter surrounded by unambiguous puncta and therefore are not considered positive for expression. Scale bar is 5 µm.

D. Counting

23 Select and display DAPI signal and only one RNAscope signal.

24 Select the Event Marker Tool under the Graphics menu in Zen.

25 Mark a nucleus with the marker tool if it meets the classification criteria above.

26 When finished counting for a given RNA signal, right click to create a counting graphic and end counting. The total number of positive cells will be displayed.

27 Rename the counting graphic if desired and hide it from view before repeating steps 1-4 for the remaining RNAscope signals (if any).

28 For assessing colocalization, display counting graphics for two or more signals and find where they overlap (Figure 4A).

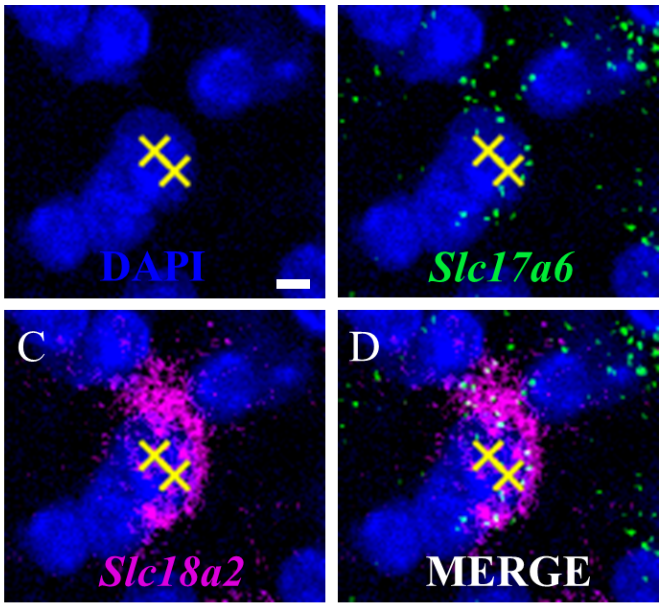

Figure 4. Co-positive expression.
A. A nucleus marked by two yellow event markers for expression of two RNAs. **B**. Confirmation nucleus is positive for the first RNA. **C**. Confirmation nucleus is positive for the second RNA. **D**. Merge image confirming colocalization of both markers. Scale bar is 5 µm.

28.1 If two or more event markers appear to be on the same nucleus, display their respective RNA signals one at a time to verify they both colocalize on the same nucleus. If they do, use the event marker tool to mark the nucleus as a co-positive cell. (Figure 4B-C)

E. Border criteria

29 If there is a cell on a single border (i.e. if one side of the border is a VTA or SNc subregion and the other side is neither VTA nor SNc), count it in the sub region if >50% of nuclei is within the sub region.

30 If there is a cell on a double border (one side is one subregion, the other side is another subregion), assign it to the subregion where >70% of the nucleus is present. If about 30-70% of the cell is on the border, assign it to the subregion that is medial of the border.

31 If the border is horizontal, then assign it to the subregion dorsal.

32 Avoid placing an event marker on top of a border, as the location of event markers are not editable after they’re placed.