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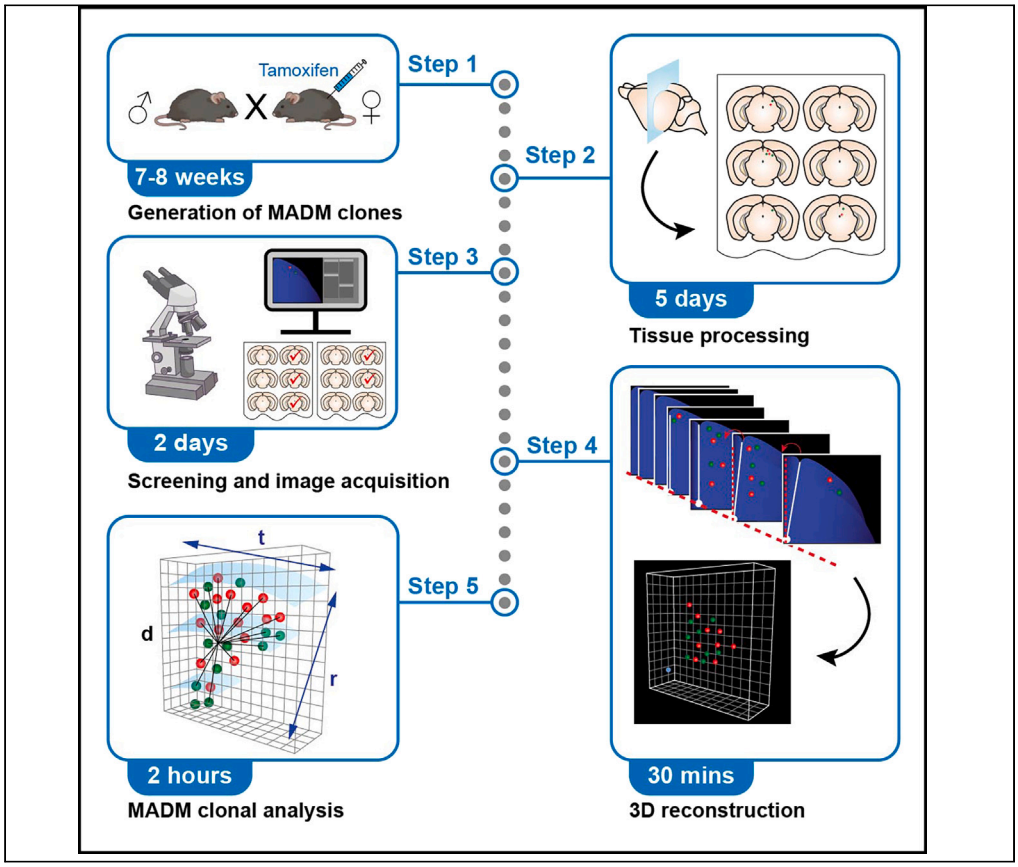
Protocol for quantitative reconstruction of cell lineage using mosaic analysis with double markers in mice



The generation of diverse cell types during development is fundamental to brain functions. We outline a protocol to quantitatively assess the clonal output of individual neural progenitors using mosaic analysis with double markers (MADM) in mice. We first describe steps to acquire and reconstruct adult MADM clones in the superior colliculus. Then we detail analysis pipelines to determine clonal composition and architecture. This protocol enables the buildup of quantitative frameworks of lineage progression with precise spatial resolution in the brain.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Experimental workflow for the generation of MADM clones to label cell lineage

Procedures to screen for and acquire MADM clones in brain tissue

Step-by-step guide to align and reconstruct individual MADM clones in 3D

Quantitative assessment of clonal composition and 3D architecture

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Protocol for quantitative reconstruction of cell lineage using mosaic analysis with double markers in mice

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SUMMARY

The generation of diverse cell types during development is fundamental to brain functions. We outline a protocol to quantitatively assess the clonal output of individual neural progenitors using mosaic analysis with double markers (MADM) in mice. We first describe steps to acquire and reconstruct adult MADM clones in the superior colliculus. Then we detail analysis pipelines to determine clonal composition and architecture. This protocol enables the buildup of quantitative frameworks of lineage progression with precise spatial resolution in the brain.

For complete details on the use and execution of this protocol, please refer to Cheung et al.[1]

BEFORE YOU BEGIN

This protocol details experimental procedures for the generation, preparation, reconstruction and analysis of individual clonally-related cluster of cells labeled using Mosaic Analysis with Double Markers (MADM).[2,3] While specific steps and examples are provided for the analysis in the mouse superior colliculus (SC), this protocol is not restricted to any brain region and can be used throughout the brain.

The precise ontogenetic programs governing neural stem cell proliferation behavior and potential has been crucial to our understanding of the development of the brain in health and disease. Here, we outline the experimental protocol, utilizing the MADM approach to fluorescently label, spatially reconstruct and analyze radial glial progenitor (RGP) cell lineage.

MADM relies on CreER-mediated interchromosomal events in dividing RGPs to differentially label daughter cells and their progeny *in vivo*.[2,3] Using cell-type specific inducible CreER-drivers, the resulting individual groups of labeled cells, also referred to as MADM clones, represent single units of RGP output. Therefore, MADM allows for precise labeling of clonally-related cells. The unique dual color system of MADM in green fluorescent protein (GFP) and tdTomato (tdT) provides an unambiguous readout of RGP division pattern. The sparse but brightly fluorescent labeling of MADM ensures precise analysis at single-cell and single-progenitor resolution.[4–9]

Depending on the induction time point, clone size can vary greatly.[1,5] Given the sparseness of labeling, we find that pre-screening serial brain sections or after clearing before reconstruction is the most time- and cost-effective option. MADM allows for better visualization of fine morphology of cells as well as the possibility to perform immunostaining of cell-type markers of interest.[3]

While we outline experimental procedures for collection and analysis of clones in adult brain tissues, our protocol is applicable to any developmental time points. For further details on general planning

of MADM mouse breeding[3,10] and for MADM clonal analysis,[11] readers are directed to our previous protocols.

Institutional permissions

This protocol involves experimentation on live mice. Permission must be obtained from relevant institutional and governmental committees before beginning with experiments. All animal procedures described in this protocol were approved by the Austrian Federal Ministry of Science and Research in accordance with the Austrian and European Union animal law (license number: BMWF-66.018/0007-II/3b/2012 and BMWFW-66.018/0006-WF/V/3b/2017).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Sigma-Aldrich	Cat# T5648
Corn oil	Sigma-Aldrich	Cat# C8267
Avertin (2,2,2-tribromoethanol)	Sigma-Aldrich	Cat# T48402
t-amylalcohol (2-methyl-2-butanol)	Sigma-Aldrich	Cat# 240486
Ketamine (100 mg/mL)	VetViva Richter	Cat# 8-01141
Xylazine (20 mg/mL)	Ogris Pharma	Cat# 8-00178
Acepromazine (10 mg/mL)	Vana	Cat# 8-00442
$NaH_2PO_4 \cdot 2H_2O$	Sigma-Aldrich	Cat# 71505
Na_2HPO_4	Sigma-Aldrich	Cat# S3264
NaOH	Carl Roth	Cat# T198.1
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat# 441244
Sucrose	Sigma-Aldrich	Cat# S8501
O.C.T. Tissue-Tek	Sakura	Cat# 4583
DAPI	Thermo Fisher Scientific	Cat# D1306
Glycerol	Sigma-Aldrich	Cat# G5516
Mowiol 4-88	Carl Roth	Cat# 0713.2
DABCO (1,4-Diazabicyclo[2.2.2]octane)	Carl Roth	Cat# 0718.2
Experimental models: Organisms/strains		
Mouse: *MADM-11-GT* both sexes; 2–8 months	The Jackson Laboratory Hippenmeyer et al.[9]	RRID:IMSR_JAX:013749
Mouse: *MADM-11-TG* both sexes; 2–8 months	The Jackson Laboratory Hippenmeyer et al.[9]	RRID:IMSR_JAX:013751
Mouse: *Fzd10-CreER* both sexes; 2–8 months	Cheung et al.[1]	N/A
Mouse: wild-type CD-1 strain for fostering females; 2–8 months	Charles River	RRID:IMSR_CRL:022
Software and algorithms		
ZEN Digital Imaging for Light Microscopy – Blue 2.3 and 2.6	ZEISS	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html; RRID:SCR_013672
Fiji (v1.53)	Schindelin et al.[12]	https://fiji.schttps://fiji.sc; RRID:SCR_002285
Microsoft Excel	Microsoft	https://www.microsoft.comhttps://www.microsoft.com; RRID:SCR_016137
Graphpad Prism 8.0	GraphPad	https://www.graphpad.comhttps://www.graphpad.com; RRID:SCR_002798
MATLAB R2022b	MathWorks	http://www.mathworks.com/products/matlab/; RRID:SCR_001622
Other		
Peristaltic pump	Watson-Marlow	Cat# 323S/D
Dissection tools – various	Fine Science Tools	https://www.finescience.com
Cryostat CryoStar NX70	Thermo Fisher Scientific	https://www.thermofisher.com
SuperFrost glass slides	Thermo Fisher Scientific	Cat# J1800AMNZ

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cover slips 24 × 50 mm	VWR	Cat# 631-0147
ZEISS Axio Imager 2 microscope	ZEISS	https://www.zeiss.com/microscopy/en/products/light-microscopes.html RRID:SCR_018876
ZEISS LSM 800 confocal microscope	ZEISS	https://www.zeiss.com/microscopy/us/local/zen-knowledge-base-home/zen-knowledge-base-lsm-800-900-980.html

MATERIALS AND EQUIPMENT

Preparation of stock solutions prior to beginning with experiments.

Tamoxifen working solution

Reagent	Final concentration	Amount
Tamoxifen	20 mg/mL	200 mg
Corn oil	N/A	10 mL
Total	**20 mg/mL**	**10 mL**

Store at 4°C for up to 2 weeks, protected from light.

⚠ CRITICAL: Tamoxifen is carcinogenic. Work under fume hood and handle with gloves.

Anesthesia stock solution

Reagent	Final concentration	Amount
Avertin	100%	7 g
t-amylalcohol	N/A	7 mL
Total	**100%**	**7 mL**

Store at 4°C for up to 6 months, protected from light.

Anesthesia working solution (2.5%)

Reagent	Final concentration	Amount
Anesthesia stock solution 100%	2.5%	0.875 mL
1× PBS	N/A	34.125 mL
Total	**2.5%**	**35 mL**

Filter and store at 4°C for up to one month, protected from light.

Ketamine/Xylazine/Acepromazine mixture

Reagent	Final concentration	Amount
Ketamine	43.5 mg/mL	5 mL
Xylazine	8.7 mg/mL	5 mL
Acepromazine	1.3 mg/mL	1.5 mL
Total	**N/A**	**11.5 mL**

Store at 4°C for up to one week.

1 M PB buffer (pH 7.4)

Reagent	Final concentration	Amount
2 M $NaH_2PO_4 \cdot 2H_2O$ (monobasic sodium phosphate)	0.19 M	47.5 mL
2 M Na_2HPO_4 (dibasic sodium phosphate)	0.81 M	202.5 mL
ddH_2O	N/A	250 mL
Total	**1 M**	**500 mL**

Store at 22–25°C.

4% PFA		
Reagent	Final concentration	Amount
Paraformaldehyde (PFA)	4%	40 g
2 N NaOH	0.008 N	4 mL
1 M PB buffer	0.1 M	100 mL
ddH_2O	N/A	850 mL
Total	**4%**	**1,000 mL**
Store at 4°C for up to 1 week.		

Note: Dissolve PFA in solution warmed up to ~55°C in a fume hood.

⚠ CRITICAL: PFA is toxic and hazardous upon direct skin contact or inhalation. Prepare 4% PFA and perform all experiments using 4% PFA under a fume hood.

30% sucrose		
Reagent	Final concentration	Amount
Sucrose	30%	300 g
1× PBS	N/A	Add up to 1,000 mL
Total	**30%**	**1,000 mL**
Filter and store at 4°C up to 1 month.		

DAPI stock solution		
Reagent	Final concentration	Amount
DAPI (1 vial)	5 mg/mL	10 mg
ddH_2O	N/A	2 mL
Total	**5 mg/mL**	**2 mL**
Store at −20°C in aliquots.		

DAPI working solution		
Reagent	Final concentration	Amount
DAPI stock solution	0.5 μg/mL	5 μL
1× PBS	N/A	50 mL
Total	**0.5 μg/mL**	**50 mL**
Store at 4°C for up to one week, protected from light.		

Mowiol-DABCO		
Reagent	Final concentration	Amount
Glycerol	N/A	6 g
Mowiol	N/A	4 g
ddH_2O	N/A	6 mL
0.2 M Tris-HCl (pH 8.5)	0.1 M	12 mL
DABCO	N/A	25 mg
Total	**N/A**	**25 mL**
Store at −20°C in aliquots.		

Note: This solution takes several hours to prepare: Mix Mowiol and Glycerol for 1 h and mix in ddH_2O for 1 h by constant stirring. Add Tris-HCl and stir for 2 h at 50°C. Finally, mix in DABCO until solution turns clear.

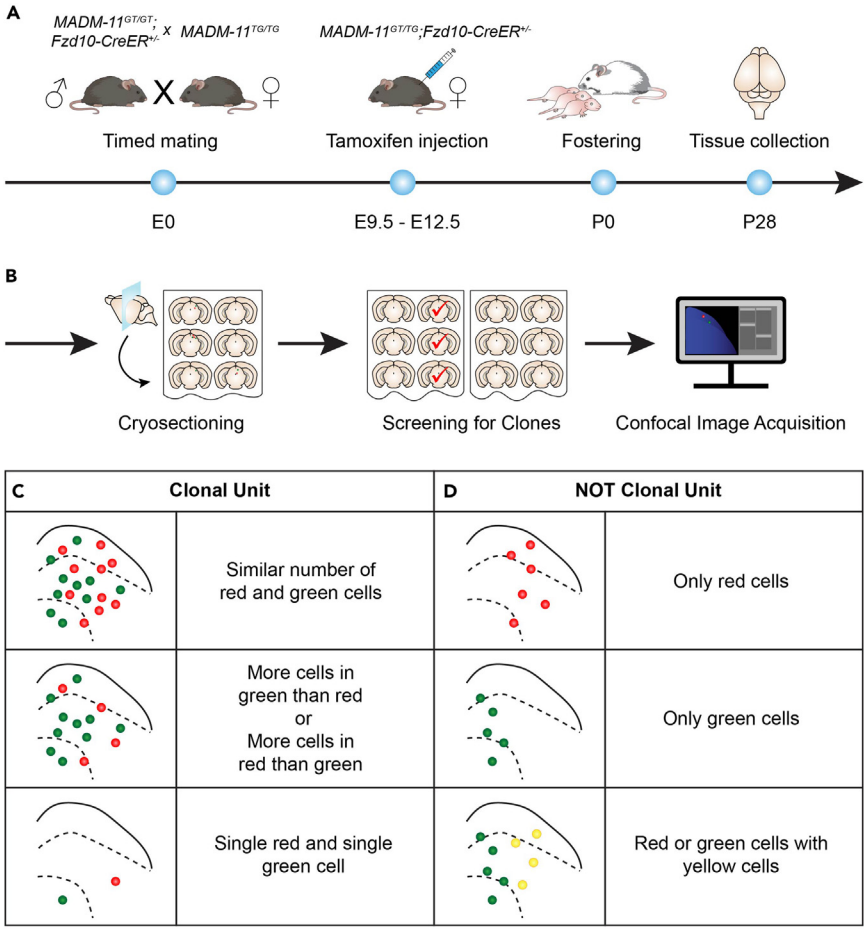


Figure 1. Workflow of the generation and identification of MADM clones

(A and B) Schematics illustrate the generation of MADM clones in the SC from timed mating to tissue collection at adult stages (A), which is followed by the processing of brains for high-quality confocal image acquisition (B).

(C and D) Schematics illustrating some examples of cell clusters which are considered as clonal units (C) and those that are not (D). Red and green cells are represented by colored spheres. The superior colliculus layers are marked by solid and dotted lines.

STEP-BY-STEP METHOD DETAILS

Generation of MADM clones

Timing: 7–8 weeks

This section describes the generation of MADM clones in the SC (Figure 1A).

1. Begin timed mating of experimental mice and foster females:
   a. Prepare foster mothers by setting up additional wild-type mating (e.g., mice of CD-1 strain) on the same day or 1 day prior to setting up experimental mating.
   b. Start experimental mating by crossing *MADM-11* $^{GT/GT}$*;Fzd10-CreER*$^{+/-}$ mice with *MADM-11* $^{TG/TG}$ mice between 2 and 8 months of age.
   c. Check for vaginal plugs in the next morning and separate plugged females. Mark the date as embryonic day (E) 0.

Note: The choice of MADM reporters and CreER-driver depends on the research question. We recommend using MADM cassettes on chromosome 11 (MADM-11) for optimal efficiency

of MADM clone induction.[5] The use of *Fzd10-CreER* to induce MADM event in progenitors in the SC has been validated.[1]

Note: The generation of *MADM-11*$^{GT/GT}$*;Fzd10-CreER*$^{+/-}$ mice should be carried out prior to the start of this protocol by crossing *MADM-11-GT* with *Fzd10-CreER* mice. *CreER* expression can be introduced to the parent mouse line with either the MADM-11-*GT* or the –*TG* cassette. However, when planning the parent mice for this cross, it is important to take into account that females cannot usually be reused for multiple breeding (see step 3).

2. Induce MADM labeling by tamoxifen (TM) administration at desired developmental stage:
   a. Define a day of induction. We found robust labeling in the SC between E9.5 and E12.5, indicating the presence of dividing progenitors during this period.[1]
   b. On the day of induction, administer TM (1–2 mg/mouse) by intraperitoneal (IP) injection on individual plugged females. troubleshooting problem 1.

Note: For analysis using other CreER-drivers or in brain regions other than the SC, it is recommended to consult relevant literatures prior to MADM experiments, to identify optimal period of proliferation. Alternatively, pilot experiments using immunostaining of classical proliferative markers (e.g. Ki67 or PAX6) can be performed over a developmental time course to assess the extent of proliferation.

⚠ CRITICAL: The exact amount of TM used should be titrated to ideally label less than one clone per brain on average. The optimal amount of TM depends on mouse strains, MADM lines, CreER-drivers, injection time points and brain regions of interest. Optimal dosage should be empirically determined for each condition. We recommend to start by generating a titration curve for 0.5 mg, 1 mg, and 2 mg TM injection per mouse at the peak time point of proliferation, to identify a dosage which provides an optimal clonal frequency. Once determined, dosage can be adjusted for other time points. In general, TM dose can be mildly increased (by 0.5–1 mg) for later developmental time points, lower expression level of CreER, or if smaller clones are expected. In contrast, dosage should be decreased (by 0.5–1 mg) towards earlier developmental time points, when higher expression level of CreER or larger clones are expected.

⚠ CRITICAL: Closely monitor the health condition of injected females over the next few days for any signs of discomfort.

3. Perform Cesarean section (C-section) to extract experimental mice for fostering:

Note: Fostering is highly recommended when TM injection is to be administered on embryonic days on or earlier than E12.5 since injected females often have problems giving birth naturally which will result in the loss of experimental mice.[11]

   a. On day E18.5-E19.5 of experimental mice, check that a foster mother which has given birth within the last 2 days is available. troubleshooting problem 2.
   b. Sacrifice TM-injected females by cervical dislocation.
   c. Perform C-section on injected female and carefully remove and clean individual pups. The following steps can enhance the survival of pups:
      i. Take care when cutting each umbilical cord to prevent excessive blood loss.
      ii. Carefully clean the nose and mouth of each pup to ensure the airways are not blocked.
      iii. Make sure that the pups are kept warm during the process by keeping them in the hands of the experimenter or using a warm surface.

d. Remove foster litter from foster mother and replace with experimental litter. The following steps can help ensure successful fostering:
   i. Clean off blood and fluid covering the experimental pups as much as possible.
   ii. Only place warm and breathing pups, which have turned pink, with the foster mother.
   iii. Rub new pups with the old bedding in the cage so that they can acquire the same scent of the foster mother and litter.

⚠ CRITICAL: Monitor the behavior of the foster mother over the next 10 min. It is a good sign of successful fostering when foster mother begins to collect new pups in a group and clean them. Continue monitoring the behavior of the foster mother and the conditions of the pups over the next few days in case of delayed rejection. troubleshooting problem 3

4. Upon successful fostering, perform standard genotyping to identify mice expressing both MADM cassettes and *CreER* before day of collection.

⚠ CRITICAL: Toe biopsies should be collected between postnatal days (P) 6 and P8. Alternatively, ear notches can be collected between P18 and P21. Adhere to institutional protocol for the collection of biopsies from mice. As a reference, see guidelines provided by *NIH Office of Intramural Research* (https://oacu.oir.nih.gov/system/files/media/file/2022-01/b3-rodent_genotyping.pdf).

Note: In case MADM cassettes are in homozygous configuration in breeding parent mice, all pups should be transheterozygous and thus have both GT/TG cassettes.[3,10] In this case, only genotyping for *CreER* is necessary.

5. Use mice at desired time point. For adult SC, we typically examine mice on P28 when the cytoarchitecture is fully established.

⚠ CRITICAL: When mice after weaning age are used, be sure to wean litter at recommended age prior to use.

Tissue collection and preparation of brain sections containing MADM clones

Timing: 5 days

This section describes the protocols to collect (Figure 1A) and process (Figure 1B) adult brains containing MADM clones in the SC.

6. On the day of sample collection, perform perfusion and collect brains:
   a. Anesthetize mice by IP injection of either anesthesia working solution (400–600 mg/kg) or a mixture of Ketamine (65 mg/kg), xylazine (13 mg/kg) and acepromazine mixture (2 mg/kg).
   b. Perform transcardial perfusion with 10 mL ice-cold PBS using a peristaltic pump (4–6 mL/min) until the liver turns pale.
   c. Continue perfusion with 10 mL ice-cold 4% PFA.
   d. Dissect the brains and postfix in 4% PFA for 16–24 h at 4°C.

Note: The concentration of anesthesia used is chosen based on guidelines for use of anesthesia (e.g. https://oacu.oir.nih.gov/system/files/media/file/2023-05/b14_pharmaceutical_compounds.pdf or https://animal.research.uiowa.edu/iacuc-guidelines-anesthesia) with appropriate adaption to the age, size and high body fat content of P28 mice.[10]

Note: Refer to our step-by-step protocol[11] for detailed experimental procedures to perform transcardial perfusion.

7. Transfer tissue to 5–7 mL of 30% sucrose solution in a 15-mL falcon tube and store upright at 4°C over 3–4 days until tissue sinks to the bottom of the tube.
8. Embed tissue in O.C.T. in a coronal orientation and store at −20°C for 3 months or at −80°C for longer term.

Note: To facilitate subsequent 3D reconstruction of clones in the SC, we recommend using coronal sections.

9. Prepare serial cryosections of the SC:
   a. Using a cryostat, collect 45 μm-thick serial coronal sections containing the SC in PBS. We routinely obtain about 50–60 sections per a P28 mouse brain.

⚠ CRITICAL: It is crucial at this stage to retain the order of brain sections and the left-right hemisphere orientation. We suggest to place single section sequentially in individual wells of 24-well plates during collection. In addition, a cut on one side of the brain away from the SC could be used to mark left-right orientation.

   b. Mount cryosections onto glass slides in order of collection and let dry.
   c. Wash slides twice with PBS for 10 min each.
   d. Incubate slides in DAPI working solution for 15 min.
   e. Cover slides using Mowiol-DABCO solution and cover with cover slips.
   f. Store at 4°C for up to 2 weeks before processing.

Pause Point: Screening of clones and image acquisition can be performed anytime within 2 weeks of mounting. Longer storage period may lead to fading of endogenous MADM labeling and DAPI signal over time.

Screening and confocal imaging of MADM clones

Timing: 2 days (depending on the number of confocal images to be acquired)

This section describes the steps to screen for and document MADM clones in the SC (Figure 1B). Due to the sparse clonal labeling of MADM, not every brain is expected to have a clone.[1] Clone size could be as low as two cells. Thus, is it important to carefully pre-screen for clones before proceeding with high-quality image acquisition.

10. Screen and document the presence of MADM clones in the SC:
    a. Using an epifluorescence microscope (e.g., Zeiss Axio Imager 2) equipped with filters for GFP and tdT and a 10× objective, screen through mounted cryosections for the presence of green or red cells.
    b. Document the presence of cells in a single or in continuous sections as a clone and note the relevant sections and the hemisphere.

Note: Yellow cells expressing both GFP and tdT can be found, but should not be used for clonal analysis. These cells could be a result of a Z-segregation or G0/G1 recombination, which in both cases do not constitute a complete clone. For more details, see previous documentations.[3]

⚠ CRITICAL: It is important at this stage to establish criteria to define a clonal unit and be consistent throughout screening. Some examples of clonal units and those not considered clonal units are illustrated in Figures 1C and 1D. According to the MADM principle, a clone should contain at least one green and one red cell in a cluster.[2,3] The number of green or

red cells may vary and does not need to be equal in each clone (Figure 1C). Single colored cell clusters or clusters containing yellow cells with either green or red cells should not be considered as clones (Figure 1D). troubleshooting problem 4 and 5

11. Acquire confocal images of selected sections containing clones with the following parameters:
    a. To aid reconstruction, always image a standardized area of each section covering the entire dorsal midbrain hemisphere. It is important to include the midline and the aqueduct (Figure 2A).
    b. Image each section as a separate stack or file. For example, a clone spanning across 3 consecutive sections should be imaged in 3 image stacks or files.

Note: As a reference, for a P28 SC hemisphere, we routinely use the following settings on a Zeiss LSM-800 inverted confocal microscope with a 10× objective: 1024 × 1024 frame size, 8 bits per pixel, 4 × 4 tile, z-stack interval of 5 μm, 9 stacks per section, image GFP, tdT and DAPI.

12. Save confocal images until further analysis.

Pause Point: Confocal images can be processed and analyzed at any time after acquisition.

3D reconstruction of MADM clones

Timing: 30 min

This section describes image processing steps where clones spanning over multiple cryosections are aligned and combined in 3D. Images are processed one clone at a time.

13. Stitch confocal image tiles using ZEN software by selecting "Stitching" option under "Processing" tab.

⚠ CRITICAL: For accurate image reconstruction, tile overlapping parameters should match between image acquisition and stitching steps. Default settings are software-dependent and should be verified prior to image processing. We routinely use default settings on ZEN software which acquires tiled images with 10% overlap and performs stitching with a minimal overlap of 5% and maximal shift of 10%. We recommend choosing "Fuse Tiles" mode.

14. Export stacks as merged channel TIFF images by selecting "Image Export" option under "Processing" tab.
15. Create a folder in the name of the clonal ID and store all exported image stacks of the relevant clone within the folder.
16. Rename each individual stack as "CloneID-XX" where XX represents section number in increasing order (e.g., CloneA-01, CloneA-02, CloneA-03, etc…).

Note: Image stacks should be in 8-bit, 3 channels with 9 slices by default. If needed, conversions can be made using "Re-order Hyperstack" under Image/Stack on FIJI.

17. Open all image stacks of the same clone on FIJI and view in "Tile" mode under "Window" tab. See Data S3.
18. For each stack, scroll to the middle of the z-stack where the DAPI signal is the strongest. Adjust brightness of DAPI signal if necessary.
19. Select the straight line tool on the tool bar, draw a straight line beginning from the aqueduct vertically upwards along the midline of the dorsal midbrain (Figure 2B).

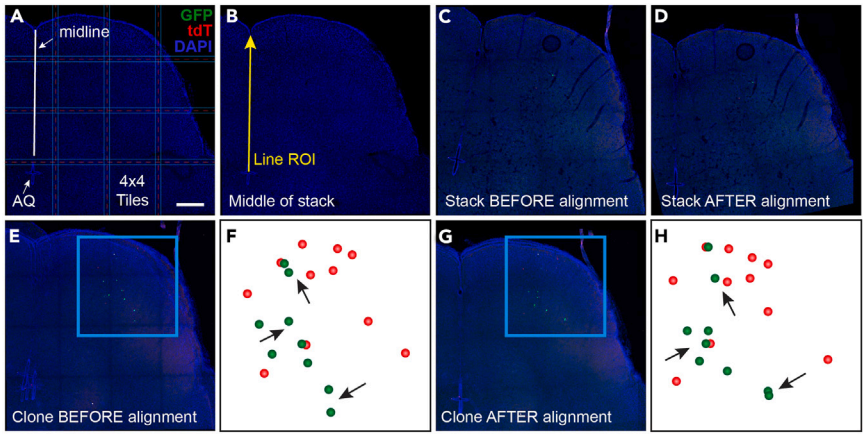


Figure 2. Image acquisition and 3D alignment of MADM clones

(A) Example of a standardized 4 × 4 tiled image of a dorsal midbrain hemisphere including the midline and the aqueduct (AQ).

(B) An image showing the middle of an image stack with a line region of interest (line ROI) drawn in the direction as shown during image alignment.

(C) A maximum z-projection of a tiled stack imaged at an angle.

(D) The same stack in (C) after image alignment.

(E–H) Maximum z-projection of a clone acquired across 5 image stacks before (E) and after (G) alignment. Schematics represent the position of cells in the clone before (F) and after (H) alignment. Red and green cells are represented by colored spheres. Arrows indicate regions where the relative positions of cells have been corrected by image alignment. Scale bar = 200 μm.

⚠ CRITICAL: The orientation of the line is important thus should always be draw from the aqueduct upwards in the case of the superior colliculus (Figures 2B and Data S3).

Note: For other brain regions, select any two constant landmarks instead and proceed accordingly. Examples of landmarks are points along midline, ventricles, and boundaries of tissue or layers that can be clearly defined and present in every stack. In this case, line ROIs are not required to be of a specific length or in a specific orientation. It is however important to keep in mind that the line should connect the first landmark to the other in the same order for every stack. Only the position of the first landmark and the angle of the line towards the second landmark are used for alignment.

20. Repeat steps 18 and 19 to each open stack to define individual line regions of interest (ROIs).
21. Drag and drop macro file "3DSerialSectionAlignmentLineROI.ijm" (Data S1) in FIJI. A separate window containing the script will open.

Note: For reference and to enable further customized adaptations, the macro script is annotated with important information and explanation.

22. Press Run. 3D alignment will proceed automatically. The results of the alignment are illustrated for a single stack (Figures 2C and 2D) and an entire clone (Figures 2E–2H). Note that the distribution of cells within the clone is corrected by alignment (Figures 2F and 2H). troubleshooting problem 6

Note: As shown in Data S3, this macro starts by rotating the first stack such that its line ROI is vertical. It then goes through subsequent stacks one-by-one to transform the marked location of the aqueduct to the same position as the first stack and rotate to straighten the midline. As a result, the following files are automatically saved in the same folder: maximum z-projections of

each aligned stack, a combined aligned stack of the entire clone and a maximum z-projection of the entire clone.

Note: Although we routinely perform alignment on image stacks containing 3 channels, this macro can be used for images containing any number of channels.

Note: While the final aligned image will always be vertical to the line ROI, it can be easily rotated if required, using "Rotate" function under Image/Transform on the final stack or projection.

23. Optionally, if clones should be visually presented in 3D, use built-in "3D Viewer" function in FIJI. Alternatively, other software like Imaris image analysis software can be used.

Quantitative analysis of MADM clones

Timing: 2 h

This section describes steps to obtain quantitative parameters and 3D architecture of each clone. We first provide steps to obtain clonal parameters such as cell number and layer distribution which allow us to compare the output of individual progenitors. Then, we outline details to additionally measure precise 3D clonal architecture such as the dispersion and distribution of cells in both radial and tangential dimension. The additional quantitative readouts in 3D provide accurate measures of the spatial relationship of clonally-related cells and enable us to make precise comparisons between clones induced across different time points. Depending on which parameters are of interest, a Microsoft Excel template can be used for data recording and analysis by applying the geometric calculations we provide below. Alternatively, other software like MATLAB can also be used. A Microsoft Excel template is provided as Data S2.

24. Extract basic parameters and 3D coordinates from each cell of a clone (Data S3):
    a. Open Microsoft Excel to record parameters of each cell. Use an empty worksheet or a predefined template (Data S2) if available.
    b. Open aligned stack of a single clone in FIJI.
    c. Open ROI manager under "Analyze" and "Tools"
    d. Choose point tool from tool bar.
    e. Scroll to the middle of the z-stack and mark by clicking on the aqueduct. This will be a reference point and the first point of each clone.
    f. Press "t" to store the coordinates of the marked position of the reference point in ROI manager.
    g. Starting from the beginning of the stack, mark the center of a cell with a click and store the coordinates by pressing "t" in ROI manager.
    h. For each cell, also record the color, cell type based on morphology, and layer position in Excel file.
    i. Repeat step 24 g-h for each cell of the clone.

Note: Based on DAPI staining, the sSC, dSC and the PAG layers can be clearly defined (Figure 3A). The bottom of sSC is marked by the optical layer (*stratum opticum*, SO), while the bottom of the dSC is marked by the white matter layer (*stratum album profundum*, SAP).

Note: Neurons, astrocytes and oligodendrocytes can be visually distinguished in the adult SC by their stereotypical morphology (Figure 3A). Astrocytes display protoplasmic morphology (Figure 3B) while oligodendrocytes are small and often appear in clusters (Figure 3C). Some distinctive cell-type specific morphology of neurons like wide-field (Figure 3D) and narrow-field (Figure 3E) cells in the sSC as described in[13] can also be observed.

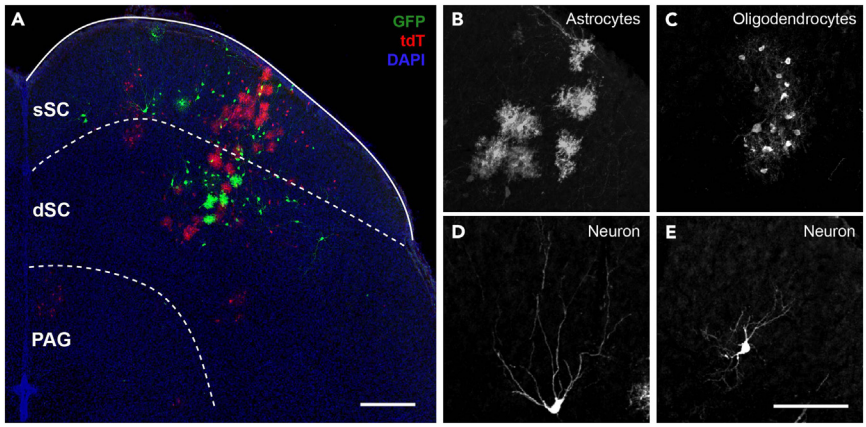


Figure 3. MADM clonal composition in the SC

(A) An example image of a MADM clone in the SC. Layers are shown with white solid and dotted lines and marked as superficial superior colliculus (sSC), deep superior colliculus (dSC) and periaqueductal gray (PAG).

(B–E) High magnification of images of astrocytes (B), oligodendrocytes (C), a wide-field neuron (D) and a narrow-field neuron (E) are shown. Scale bars = 200 μm in (A) and 50 μm in (B-E).

j. After marking every cell of the clone, save ROIs under ROI manager.

k. Open "Set Measurements" under "Analyze" tab and uncheck all boxes leaving only "Invert Y coordinates" checked and press "OK".

Note: The "Invert Y coordinates" selection is required since, by default, the (0,0) x-y coordinate is assigned to the top left corner of an image in FIJI. This should be verified if other software is used. It is not necessary to check this option if the default (0,0) coordinate is at the bottom left corner instead.

l. Extract coordinates of each cell by pressing "Measure" in ROI manger. A separate window will open.

m. Copy and paste x-, y-, z- coordinates of each cell to the Excel file. Note that x- and y- are measured in pixel number and z- in slice number.

25. Convert x- and y- coordinates to μm based on pixel size of confocal acquisition. Adjust conversion values in Cells B11 and B13 of the excel template according to image acquisition settings. 0.624 μm/pixel is specific for Zeiss LSM inverted microscopes using 10× objection.

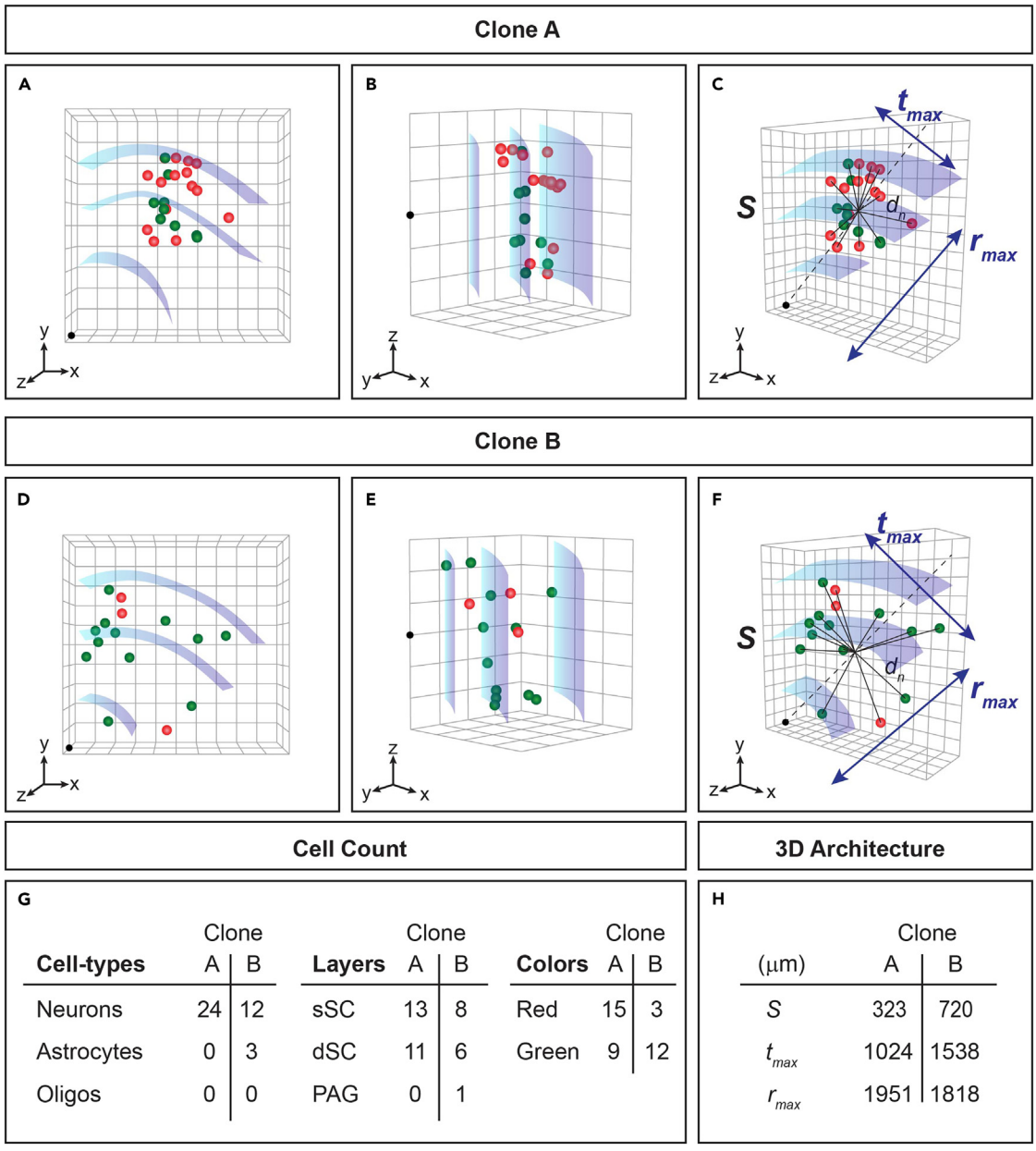
26. Determine the z- coordinates by converting slice number into μm given that z-sampling size is 5 μm. Adjust conversion value in Cell B15 accordingly if this is not the case.

Note: the accuracy of z-coordinates can be further enhanced by sampling more frequently. However, we find that 5 μm is sufficient as it is smaller than the size of typical cell body.

27. Z-projected or 3D representation of the clone can be plotted using GraphPad Prism or MATLAB software, respectively, using extracted 3D coordinates (Figure 4; Data S2, Columns L-N).

28. Compute basic parameters of each clone for comparison, for example:
    a. Cell count: count the number of red, green or total number of cells per clone (Data S2, Cells B2-B4).
    b. Layer distribution: count the proportion of cells in each layer (Data S2, Cells B5-B7).

29. For more advanced comparisons, some 3D architectures of clones can also be determined following these steps:
    a. Set x-, y- and z- coordinates of reference point to $(0, 0, 0)$ and adjust the coordinates of every cell accordingly by computing the difference between each point to reference point in each dimension (Data S2, Columns O-Q).



Cell-types	Clone A	Clone B
Neurons	24	12
Astrocytes	0	3
Oligos	0	0

Layers	Clone A	Clone B
sSC	13	8
dSC	11	6
PAG	0	1

Colors	Clone A	Clone B
Red	15	3
Green	9	12

(μm)	Clone A	Clone B
S	323	720
t_{max}	1024	1538
r_{max}	1951	1818

Figure 4. Analysis of clonal composition, distribution and 3D architecture

(A–F) Scatter plots of two 3D reconstructed clones are shown (A-C; Clone A and D-F; Clone B). Clones are viewed in 3D from the x-y (A and D) and the z-x (B and E) planes. Parameters for 3D analysis are marked as distance from centroid (d_n), dispersion (S), tangential dimension (t_{max}), and radial dimension (r_{max}) in (C and F). Blue dividing planes mark the separation between layers.

(G and H) A list of cell counts (G) and 3D architecture (H) parameters measurable in each clone for comparison.

b. Determine the coordinates of the centroid of the clone (x_c, y_c, z_c) which is the mean value of x-, y- and z- coordinates of all cells (Data S2, Cells B18-B20).

c. Determine the distance of each cell from the centroid (d_n) using the following equation (Data S2, Column R):

$$d_n = \sqrt{(x_n - x_c)^2 + (y_n - y_c)^2 + (z_n - z_c)^2}$$

where x_c, y_c, z_c = coordinates of centroid
x_n, y_n, z_n = coordinates of any cell

d. Determine the dispersion (S) of the clone using the following equation (Figures 4C and 4F) (Data S2, Cell B23):

$$S = \sqrt{\frac{\sum_{n=1}^{N} (d_n)^2}{N}}$$

where d_n = distance of any cell from centroid
N = number of cells in each clone

e. Determine the closest tangential distance (t_n) of each cell from a hypothetical midline passing through the reference point and the centroid (Data S2, Column T):

$$t_n = \sqrt{\frac{(x_n y_c - x_c y_n)^2}{x_c^2 + y_c^2} + (z_n - z_c)^2}$$

where x_c, y_c, z_c = coordinates of centroid
x_n, y_n, z_n = coordinates of any cell

f. Determine the maximum tangential dimension (t_{max}) of each clone = twice the maximum tangential distance recorded in the clone (Figures 4C and 4F; Data S2, Cell B26).

g. Determine the radial distance (r_n) between each cell and reference point using the following equation (Data S2, Column U):

$$r_n = \sqrt{x_n^2 + y_n^2 + z_n^2}$$

where x_n, y_n, z_n = coordinates of any cell

h. Determine the maximum radial dimension (r_{max}) of each clone = the maximum radial distance recorded in the clone (Figures 4C and 4F; Data S2, Cell B29).

EXPECTED OUTCOMES

The outcome of this protocol includes both qualitative 3D reconstruction and quantitative analysis of MADM clonal architecture (Figure 4). By comparing between clones generated across different time points, the output potential and division pattern of RGPs can be determined. A list of example basic parameters recorded from two different clones are listed in Figures 4G and 4H. Clone A was induced at E10.5 where B was induced one day later at E11.5. The cell count of specific cell types reveals the relative neurogenic and gliogenic potential of SC RGPs. The cell count in specific layers across different time points would reflect the order of layer production. In addition, MADM uniquely marks the division pattern of the SC RGPs by the relative number of red versus green cells. In our examples, Clone B originated from an RGP that is also gliogenic, underwent a more asymmetric division which generated cells in all 3 layers compared to Clone A. Upon precise measurements of the position of each cell in 3D space, fine details of clonal 3D architecture like the dispersion as well as tangential and radial dimensions can also be compared. In our examples, Clone A is more dispersed, wider but has a similar radial dimension as Clone A. These parameters give important insights into the coverage of individual RGPs in space. For more details on how these parameters are comparatively assessed, we refer readers to the original paper.[1]

LIMITATIONS

The power of MADM clonal analysis relies largely on the high probability of sparsely labeling single clonal units in particular brain regions. As a result, a significant number of mice are required to obtain enough clones for analysis. However, time can be saved by generating and processing brains in batches. TM injection at the optimal dose followed by successful fostering could also enhance the efficiency of this protocol. The "bottleneck" of this protocol is at the stage of confocal acquisition. To speed up this process, faster scanning systems like spinning disk confocal microscopes and the use of automated tiled sequence image acquisition can be used. In addition, available space within

the animal facility for MADM mouse breeding must be arranged before the start of experiments. These aspects should be taken into consideration when planning experiments.

TROUBLESHOOTING

Problem 1

Problems with TM injection and adverse effects observed in injected females.

Potential solution

IP injection may sometimes lead to mild soreness of the injection area which should not be long lasting. To avoid directly injecting into embryos or internal organs of the mice, slightly tilt the mouse on one side. Due to the high viscosity of corn oil, it is important to inject slowly keeping a firm hold of the mouse.

Problem 2

Foster mother has not given birth yet or is not available.

Potential solution

Wait one more day or allow the injected female to give birth naturally while monitoring its health condition.

Problem 3

Foster mother are not responsive to new litter, even after 30 min.

Potential solution

With clean gloves on, scatter the pups around throughout the cage. Scattered pups often trigger an instinctive behavior of lactating mice to gather and clean their newborns, which may promote maternal caring. If females continue to be unresponsive, transfer to a different foster mother if available.

Problem 4

Potentially more than one clone found per brain.

Potential solution

In our experience, clones do not typically cross hemispheres. Thus, when two clusters are found with one on each hemisphere, they should be considered separate clones. When more than one cluster is found on the same hemisphere, we use a minimum distance of separation of 300 μm to define two separate clones.

Problem 5

Routinely obtaining no or too many clones per brain.

Potential solution

If consistently no clones are found, check the genotypes of the parents and offspring. If the genotypes are correct, repeat with increased concentration of TM. If most brains contain more than one clone, lower the concentration of TM.

Problem 6

Rotation of stacks during alignment cuts out a corner of an area of interest.

Potential solution

This can be prevented by first increasing the canvas size of the relevant stack before alignment in FIJI under "Image" and then "Adjust" tab. Then, proceed from drawing line ROIs.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Simon Hippenmeyer (simon.hippenmeyer@ist.ac.at).

Technical contact

Additional requests regarding technical details should be directed to the technical contact, Giselle Cheung (giselle.ty.cheung@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No dataset was generated while composing this protocol. A FIJI macro script "3DSerialSectionAlignmentLineROI.ijm" and a Microsoft Excel template used in this protocol are provided as supplementary materials.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103157.

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AUTHOR CONTRIBUTIONS

G.C. and S.H. conceived and wrote the manuscript. All authors contributed to optimizing this protocol and proofread the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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