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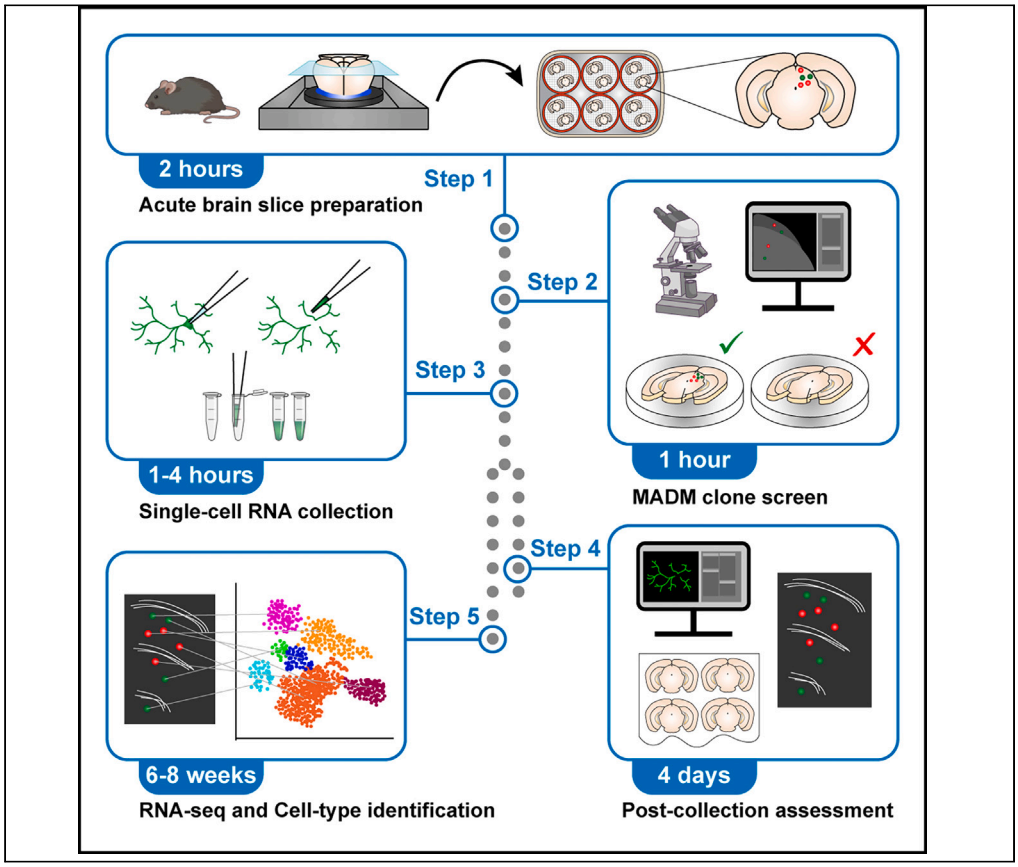
Protocol for mapping cell lineage and cell-type identity of clonally-related cells *in situ* using MADM-CloneSeq



The lineage relationship of clonally related cells offers important insights into the ontogeny and cytoarchitecture of the brain in health and disease. Here, we provide a protocol to concurrently assess cell lineage relationship and cell type identity among clonally related cells *in situ*. We first describe the preparation and screening of acute brain slices containing clonally related cells labeled using mosaic analysis with double markers (MADMs). We then outline steps to collect RNA from individual cells for downstream applications and cell type identification using RNA sequencing.

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

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Highlights

Step-by-step guide including detailed experimental workflow for MADM-CloneSeq

Procedures to generate and efficiently screen for MADM clones in acute brain slices

Experimental procedures to perform visually guided single-cell RNA collection

Post-collection assessment of clones, downstream RNA sequencing, and analysis

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Protocol for mapping cell lineage and cell-type identity of clonally-related cells *in situ* using MADM-CloneSeq

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SUMMARY

The lineage relationship of clonally-related cells offers important insights into the ontogeny and cytoarchitecture of the brain in health and disease. Here, we provide a protocol to concurrently assess cell lineage relationship and cell-type identity among clonally-related cells *in situ*. We first describe the preparation and screening of acute brain slices containing clonally-related cells labeled using mosaic analysis with double markers (MADM). We then outline steps to collect RNA from individual cells for downstream applications and cell-type identification using RNA sequencing.

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 cell-type identification of individual clonally-related 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.

We use mosaic analysis with double markers (MADM) to fluorescently label and trace the lineage of cells at single-cell and individual-progenitor resolution.[2–7] SC-specific lineage is labeled by an inducible CreER-driver, *Fzd10-CreER*.[1] The choice of CreER-drivers should be adapted for other brain regions. Taking advantage of the high level of precision and the ability to visually locate individual live cells, we have developed a method, called MADM-CloneSeq to collect RNA from individual cells for the determination of their cell-type identity.[1] The unprecedented benefit of using MADM-CloneSeq lies in the ability to extract spatial, morphological and transcriptomic profiles of each clonally-related cell.

Our protocol uses standard slice electrophysiology equipment for collection and is adapted from a Patch-seq protocol.[8] In order to increase sampling coverage of complete clones, our protocol is optimized for speed by omitting the electrophysiological recording steps. To concurrently perform whole-cell recordings, an adjustment to the internal solution is required. However here, priority is given to maximizing clonal coverage in order to accurately establish the link between clonality and cell-type identity.

The breeding and labeling of mice containing MADM clones is not covered by and should be carried out prior to this protocol. Details on the generation of MADM clones[6] and information on MADM mouse breeding,[3,9] are described in previous protocols.

Considerations for eventual cDNA library preparation approaches

The final section of this protocol describes cDNA library preparation and sequencing of collected single-cell RNA samples. With the advances in RNA sequencing methods, plenty options to produce high-quality data exist. In this protocol, we offer three options to generate cDNA libraries for sequencing, based on the availability of sequencing facilities, financial, and other factors. Upon collection, RNA samples can be submitted directly to a facility for cDNA library preparation and RNA sequencing (Option 1). Alternatively, cDNA library preparation can be performed in the laboratory using either commercial kits (Option 2) or homemade reagents (Option 3) before sequencing the cDNA libraries at a facility. A decision on which option to take should be made prior to beginning RNA sample collection. It is also important to discuss details of cost, sample storage and transfer conditions, as well as submission requirements with a designated sequencing facility before the start of experiments. We provide the following table as guidance.

	Option 1	Option 2	Option 3
cDNA preparation methods	**Sequencing facility** (e.g., Smart-Seq v2[10] or Smart-Seq v3[11])	**Commercial kits** (e.g., SMART-Seq Single Cell PLUS kit)	**Homemade reagents** (e.g., Picelli et al., 2014,[10] Cadwell et al., 2017[8] and Hagemann-Jensen et al., 2020[11])
Optimization required	**Minimal** Follow submission requirements set out by facility	**Minimal** Follow manufacturers' instructions	**Extensive** Preparation and testing of homemade reagents required
Risk of low sample quality, yield and clonal coverage	**High** Multiple transferring and handling steps of RNA	**Low** Processed in-house Minimal or no transferring step of RNA	**Low** Processed in-house Minimal or no transferring step of RNA
Storage time before processing	**Unpredictable** Likely proceeded in batches of (48–96)	**Minimal** Proceeded in small sample size (8–12)	**Minimal** Proceeded in small sample size (8–12)
Suitability for troubleshooting, trial runs, and quality control	**Not suitable** Batch processing and long processing time	**Not suitable** Costly	**Ideal** Low cost option to assess cDNA library quality prior to sequencing
Cost	**$** ~20 euros/library	**$$$** ~80–100 euros/library (SMART-Seq Single Cell PLUS kit)	**$** ~20 euros/library[8]
Ideal sample size	**>100** Based on batch processing and higher risk of reduced RNA quality	**<50** Based on high cost	**<100** Based on extensive bench work required

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
Antibodies		
GFP–chicken; 10 mg/mL; use 1:500 dilution	Aves Labs, Inc.	Cat# GFP-1020; RRID:AB_10000240
tdTomato–goat; 3 mg/mL; use 1:500 dilution	Sicgen Antibodies	Cat# AB8181-200; RRID:AB_2722750
Anti-chicken-Alexa 488; 1.5 mg/mL; use 1:500 dilution	Jackson ImmunoResearch Labs	Cat# 703-545-155; RRID:AB_2340375
Anti-goat-Alexa 568; 2 mg/mL; use 1:500 dilution	Thermo Fisher Scientific	Cat# A11057; RRID:AB_2534104
DAPI; 10 mg (reconstituted to 5 mg/mL); use 1:1,000 dilution	Thermo Fisher Scientific	Cat# D1306; RRID:AB_2629482
Chemicals, peptides, and recombinant proteins		
Avertin (2,2,2 tribromoethanol)	Sigma-Aldrich	Cat# T48402
t-Amylacohol (2-methyl-2-butanol)	Sigma-Aldrich	Cat# 240486

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
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
DEPC (diethyl pyrocarbonate)	Sigma-Aldrich	Cat# D5758
Mowiol 4-88	Carl Roth	Cat# 0713.2
DABCO (1,4-diazabicyclooctane)	Carl Roth	Cat# 0718.2
RNase Away	Thermo Fisher Scientific	Cat# 10328011
RNase inhibitor	Takara Bio	Cat# 2313A
DPBS (Dulbecco's phosphate-buffered saline)	Thermo Fisher Scientific	Cat# 14190094
Triton X-100	Thermo Fisher Scientific	Cat# 85111
Donkey serum	Thermo Fisher Scientific	Cat# 566460
Critical commercial assays		
SMART-Seq Single Cell PLUS Kit, 48 rxns	Takara Bio	Cat# R400750
Experimental models: Organisms/strains		
Mouse: *MADM-11-GT* Both sexes; 2–8 months	The Jackson Laboratory Hippenmeyer et al.[4]	RRID:IMSR_JAX:013749
Mouse: *MADM-11-TG* Both sexes; 2–8 months	The Jackson Laboratory Hippenmeyer et al.[4]	RRID:IMSR_JAX:013751
Mouse: *Fzd10-CreER* Both sexes; 2–8 months	Cheung et al.[1]	N/A
Software and algorithms		
Micromanager for electrophysiology setup	Micromanager	https://micro-manager.org/; RRID:SCR_016865
Fiji (v1.53)	Schindelin et al.[12]	https://fiji.sc/; RRID:SCR_002285
ZEN digital imaging for light microscopy – Blue 2.3 and 2.6	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html#introduction RRID:SCR_013672
Scripts for RNA sequencing data processing and analysis	Cheung et al.[1]	https://github.com/fpauler/Multipotent-Progenitors-Instruct-Ontogeny-of-the-Superior-Colliculus https://doi.org/10.5281/zenodo.10089056 (https://zenodo.org/records/10089056)
Other		
Carbogen gas (95% O_2 and 5% CO_2)	Westfalen Austria GmbH	Cat # A03660150
Plastic tubings for perfusion and carbogenation	Qosina	Various types and sizes
Plastic tubing connections for perfusion and carbogenation	Proliquid	Various types and sizes
Ceramic air diffuser for carbogenation of solutions	Hobby	Cat# DH008
3D printed multi-well slice recovery chamber (SMASH)	Printables (3D model 361319)	https://www.printables.com
Peristaltic pump	Watson-Marlow	Cat# 323S/D
Dissection tools – various	Fine Science Tools	https://www.finescience.com
Vibratome (VT1200)	Leica Biosystems	https://www.leicabiosystems.com RRID:SCR_020243
Loctite glue	Henkel Adhesives	Cat# 401
LNscope 240XY	Luigs & Neumann	https://www.luigs-neumann.org/
Orca Spark camera	Hamamatsu	https://www.hamamatsu.com/Cat# C11440-36U
pE-300 white light source	CoolLED	https://www.coolled.com/
GFP/DsRed Dualband HC filter set	Olympus	https://www.olympus-lifescience.com/
4×, 20×, 60× objectives on electrophysiology setup	Olympus	https://www.olympus-lifescience.com/UPLFLN4X, UMPLFLN20XW, LUMPLFLN60XW
LN Mini Compact manipulator	Luigs & Neumann	https://www.luigs-neumann.org/
Sutter P-1000 micropipette puller	Sutter Instrument	https://www.sutter.com/MICROPIPETTE/p-1000.html RRID:SCR_021042
Glass capillaries B150-86-10	Sutter Instrument	https://www.sutter.com/MICROPIPETTE/glass.html
Pipette microloaders	Eppendorf	Cat#EPE5242956003
Superfrost glass slides	Thermo Fisher Scientific	Cat#J1800AMNZ
Coverslips 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

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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
NovaSeq 6000	Illumina	https://www.illumina.com/systems/sequencing-platforms/novaseq.html RRID:SCR_016387

MATERIALS AND EQUIPMENT

Preparation of solutions prior to the day of experiments.

Anesthesia stock solution

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

Store at 22–25°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.

1X PBT

Reagent	Final concentration	Amount
1× PBS	N/A	247 mL
Triton-X	1.2%	3 mL
Total	**1.2%**	**250 mL**

Store at 22–25°C for up to 3 months.

Blocking solution for immunostaining

Reagent	Final concentration	Amount
1× PBT	N/A	9.5 mL
Donkey Serum	5%	500 µL
Total	**5%**	**10 mL**

Store at 4°C for up to 1 week.

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 up to 6 months.

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 up to 6 months.

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.

DEPC-treated H_2O

Reagent	Final concentration	Amount
DEPC	0.01%	2 mL
dH_2O	N/A	2,000 mL
Total	**0.01%**	**2,000 mL**

Store at 22–25°C for up to 3 months.

Note: Mix DEPC well by shaking bottle until no DEPC droplets are visible. Leave at 22–25°C for 2 h. Autoclave water to deactivate DEPC before use.

10× Krebs solution

Reagent	Final concentration	Amount
NaCl	1180 mM	68.91 g
KCl	25 mM	1.864 g
$MgSO_4 \times 7H_2O$	15 mM	3.618 g
$NaH_2PO_4 \times H_2O$	12.5 mM	1.725 g
Sucrose	350 mM	119.81 g
Myo-Inositol	30 mM	5.41 g
DEPC-treated H_2O	N/A	Up to 1 L
Total	**10×**	**1 L**

Filter and store at 4°C for up to 1 week.

NaH_2CO_3		
Reagent	Final concentration	Amount
$NaHCO_3$	1 M	21 g
DEPC-treated H_2O	N/A	Up to 250 mL
Total	**1 M**	**250 mL**
Filter and store at 22–25°C for up to 1 week.		

$CaCl_2$		
Reagent	Final concentration	Amount
$CaCl_2$	1 M	5.55 g
DEPC-treated H_2O	N/A	Up to 50 mL
Total	**1 M**	**50 mL**
Filter and store at 22–25°C for up to 1 week.		

Glucose		
Reagent	Final concentration	Amount
Glucose	1 M	45.04 g
DEPC-treated H_2O	N/A	Up to 250 mL
Total	**1 M**	**250 mL**
Filter and store at 4°C for up to 1 week.		

Pretreatment of glass pipettes before start of experiments.

- Pretreat glass pipettes by soaking in DEPC in dH_2O (1:1000 dilution) for 4 h at 22–25°C. Heat inactivate and dry glass pipettes by autoclaving.

Preparation of fresh solutions on the day of experiments.

1× ACSF		
Reagent	Final concentration	Amount
10× Krebs	1×	100 mL
1 M Glucose	0.01 M	10 mL
1 M $NaHCO_3$	0.03 M	30 mL
1 M $CaCl_2$	0.001 M	1 mL
DEPC-treated H_2O	N/A	Up to 1 L
Total	**1×**	**1 L**
Store with constant carbogenation with carbogen gas (95% O_2 and 5% CO_2) at 22–25°C. Prepare fresh on day of experiment. Ice-cold ACSF can be stored for up to 1 week. **Note:** Carbogenate solution to pH of 7.45–7.35 before adding $CaCl_2$ to prevent precipitation.		

Parameters for pipette puller (Sutter P-1000).

Parameters for pipette puller (Sutter P-1000)				
Line	Heat	Pull	Vel.	Time
1 × 1	528	0	50	250
2 × 3	515	0	12	250
3 × 1	506	0	22	250
An example of parameters used to generate glass pipette opening of about 3–5 μm.				

STEP-BY-STEP METHOD DETAILS

Preparation of acute brain slices containing MADM clones

Timing: 2 h

This section describes how acute brain slices containing MADM clones in the SC are prepared with emphasis on keeping slices in serial order and left-right hemisphere orientation. The composition of ACSF, specimen orientation and sectioning angle should be adjusted according to the age of the mice and brain region of interest. Adjustments of sodium and sucrose content as well as using sodium ion substitutes such as N-methyl-D-glucamine (NMDG) and glycerol in the ACSF cutting solution have been used to preserve slice quality and cell survival in aged mice.[13] We recommend consulting the literature for slice electrophysiology studies on specific brain region of interest.

1. Prepare solutions and equipment:
   a. Make 1 L of fresh 1× ACSF in DEPC-treated water. Keep at 22–25°C with constant carbogenation.
   b. Prepare 200 mL of 1× ice-cold ACSF on ice for perfusion with constant carbogenation.
   c. Fill multi-well slice recovery chamber with fresh 1× ACSF and pre-warm to 35°C in a water bath. Maintain constant carbogenation.

   Note: Although any commercially available multi-well recovery chamber can be used, we find that this 3D printed recovery chamber (Figures 1A and 1B) is a low-cost alternative that allows us to maintain slices in good quality while keeping the order of collection. The 3D printing files are freely available and can be downloaded from Printables (http://www.printables.com; model number 361319).

   d. Prepare dissection tools and clean with ethanol.
   e. Prepare vibratome with clean razor blade and pre-cooled buffer tray.
2. Anesthetize mice by intraperitoneal (IP) injection of anesthesia working solution (400–600 mg/kg).

   Note: Alternatively, a mixture of ketamine (65 mg/kg), xylazine (13 mg/kg) and acepromazine (2 mg/kg) can be used. The recommended concentration of anesthesia is based on animal experimentation guidelines and adapted to the age, size and high body fat content of P28 mice[9] (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).

   ⚠ CRITICAL: Always check for unresponsiveness by pinching the paws of the anesthetized mice before proceeding with perfusion. Inject a small amount of anesthesia working solution in addition if required.

3. Perform transcardial perfusion with 10–15 mL of ice-cold ACSF using a peristaltic pump (6–8 mL/min) until liver turns pale.

   Note: See Step-by-step protocol[6] for detailed experimental procedures of transcardial perfusion. Note that instead of PBS followed by 4% PFA, only ice-cold ACSF perfusion is necessary here. A successful perfusion should take no longer than 2 min. Troubleshooting problem 1.

4. Dissect and extract mouse brain taking care not to damage region of interest.
5. Keep brain in ice-cold 1× ACSF and let equilibrate for about 1 min.

   Note: A successfully perfused brain should be pale without visible blood vessels or red/pink patches.

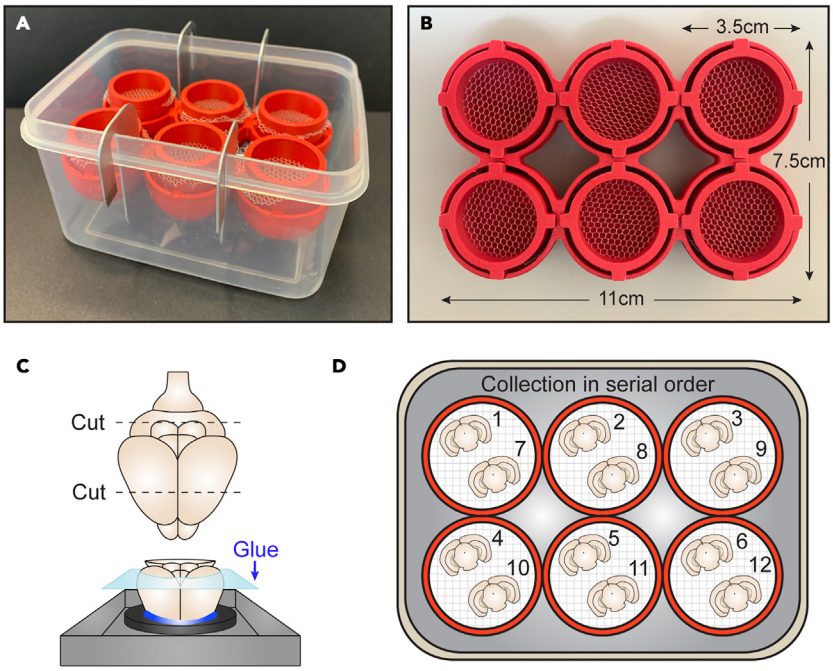


Figure 1. Serial collection of acute brain slices containing MADM clones

(A and B) Multi-well slice recover chamber with 6 collection wells (A) also shown in top down view with dimensions (B).

(C) Schematics illustrating the orientation of brain specimen and sectioning plane on the vibratome.

(D) Schematics illustrating serial collection of acute brain slices to effectively keep the order of brain slices (i.e., from 1–12).

6. Prepare brain for vibratome sectioning:
   a. Place brain on a filter paper and trim both the caudal and rostral ends of the brain using a clean razor blade as shown (Figure 1C).
   b. Glue caudal surface directly onto the middle of specimen holder.

   Note: Any type of cyanoacrylate-based glue (e.g. Loctite 401) can be used. Only a thin layer of glue should be used to prevent excess glue from creeping up and adhering to the surface of the brain.[14] Tissue that has come into contact with the glue should not be used for experiments as the glue affects cell survival and changes the consistency of the brain for accurate sectioning.

   c. Fill buffer tray with ice-cold 1× ACSF until the entire brain is covered.
   d. Make a cut in the ventral region of the brain in one hemisphere using a clean razor blade to mark left-right hemisphere orientation.
   e. Cut 200 μm-thick coronal slices in ice-cold 1× ACSF at speed of 0.08 mm/s and amplitude of 1 mm.
   f. Collect slices containing the SC in serial order in slice recovery chamber.

   Note: We recommend starting by putting one slice per well and repeating again from the first well (Figure 1D). For reference, we routinely obtain 10–12 acute slices containing the SC from an adult brain.

   g. Allow slices to recover at 35°C for 20 min and then cool to at 22–25°C before single-cell RNA collection.

Note: To do this, we recommend turning off the heating control on the water bath 20 min after collecting the last slice and allowing the bath to gradually cool to 22–25°C. Screening for clones can start during this recovery period.

Screening for MADM clones in acute brain slices

Timing: 1 h for screening 10–12 acute slices from one mouse

This section describes a critical part of MADM-CloneSeq where acute brain slices are screened for the presence of MADM clones in the SC. Due to the sparse labeling of MADM, not every brain contains a clone. A typical MADM clone can span across 2–3 consecutive acute slices. Pre-screening can greatly shorten the duration of sample collection and ensure optimal slice quality.

7. During the recovery period, individual slices should be screened for clones under a 4× or 10× objective. This can be done under the electrophysiology setup equipped with filters for green fluorescent protein (GFP) and tdTomato (tdT) or other fluorescence microscopes.

Note: Since acute slices are thick, both surfaces of each slice should be screened carefully for fluorescent cells (Figure 2). To pick up and flip or transfer acute slices, we recommend using a simple suction tool that can be made by breaking off the thin end of a glass Pasteur pipette and attaching the break point on the thicker side with a bulb to control movement of slices through the wider end of the pipette.

⚠ CRITICAL: In order to minimize cell death in acute slices, screening is most ideally performed in fresh 1× ACSF with constant carbogenation. It is however possible to briefly keep slices for not more than 5 min under a fluorescence microscope in a petri-dish containing freshly carbogenated ACSF (without constant carbogenation).

8. Discard acute slices without detectable cells.
9. If clones are found, acquire a low magnification image for documentation (Figure 2).

Note: These images will also be useful when locating the cells at 63× objective during sample collection and during post-collection reconstruction.

⚠ CRITICAL: It is important at this stage to establish criteria to define a clonal unit and be consistent throughout screening. According to the MADM principle, a clone should contain at least one green and one red cell in a cluster.[2,3] Cells appearing in yellow due to expression of both GFP and tdT can sometimes be found. However, since their clonal relationship is difficult to determine, they should be excluded from sampling. For more details, see.[3] Troubleshooting problems 2 and 3.

⚠ CRITICAL: Individual clones could span across multiple slices, be on either or both surface of a slice as well as either hemisphere. Since clones do not span from one hemisphere to the other, the left-right hemisphere distinction as well as the order of the collection can be used to identify clonal units (Figures 2Q and 2R). For example, clone A (marked by pink box) is centered in slice 2 (most cells in this slice and on both surface of the slice) and is on the opposite hemisphere of the cut. It is clearly distinct from clone B (marked by light blue box) which is on the same hemisphere of the cut. Thus, it is important to retain the order and orientation of relevant slices before, during and after screening. Keep a good record and documentation at this screening stage to improve the efficiency of subsequent collection steps.

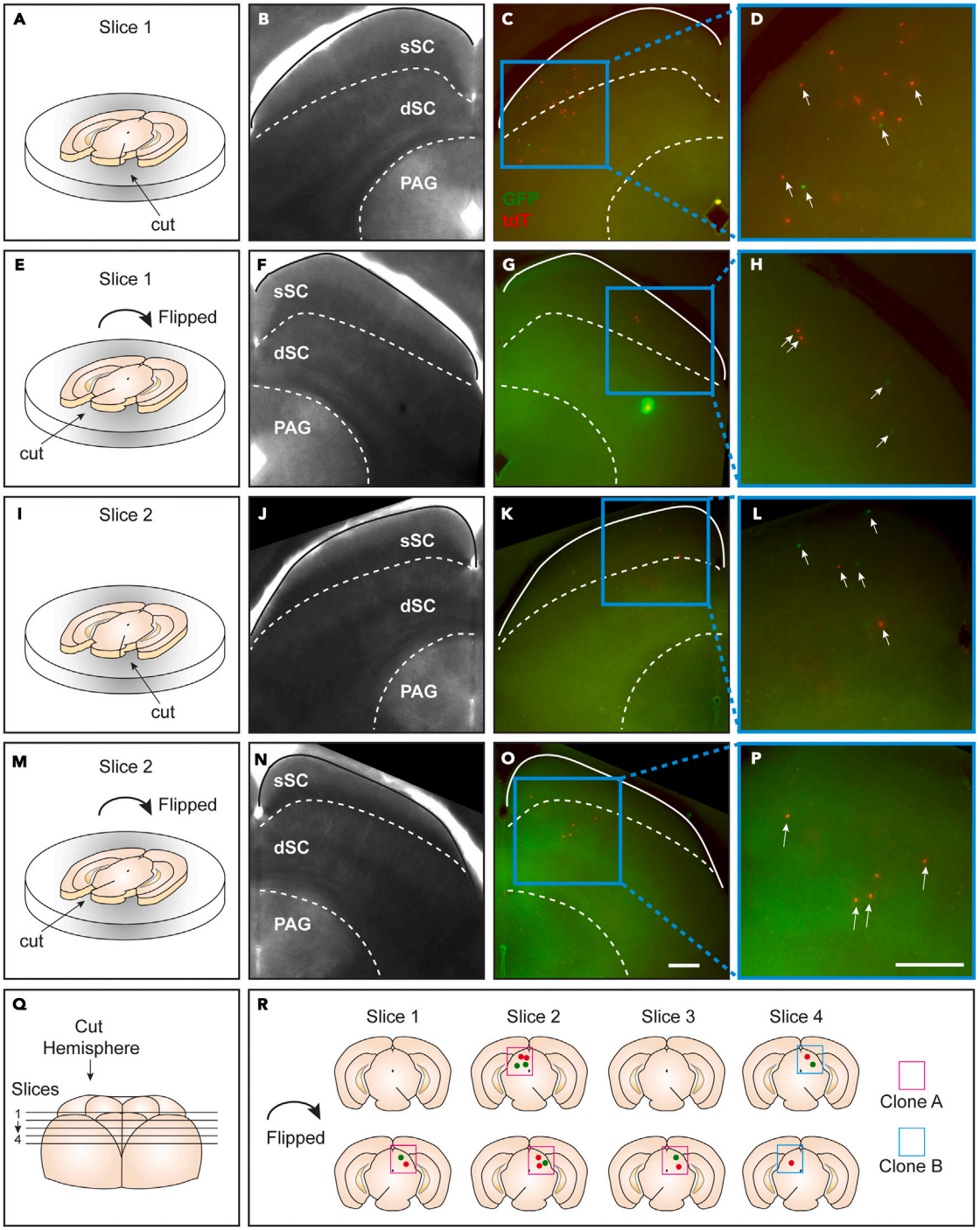


Figure 2. Screening for MADM clones in acute brain slices before RNA collection

(A–P) Schematics and images are shown for the screening of 2 brain slices where MADM-labeled cells are found on both sides of each slice. Slice 1 (A–H) and slice 2 (I–P).

(A, E, I and M) Schematics of slice orientation. Note the cut made on one side of the slice can be used to keep the left-right hemisphere orientation. (B, F, J and N) Bright field images of slices. (C, G, K and O) Fluorescence images in green (GFP) and red (tdT) showing the presence of MADM-labeled cells. (D, H, L and P) Higher magnification boxed images of (C, G, K and O).

(Q and R) Schematics showing how the order or collection and the cut made in one hemisphere (Q) can be used to distinguish two separate clones in the same brain (R; Clone A, pink box; Clone B, light blue box). Layers are separated by lines and marked as superficial superior colliculus (sSC), deep superior colliculus (dSC) and periaqueductal gray (PAG). White arrows point to MADM-labeled cells. Scale bar = 200 μm.

10. After screening, return checked slices containing clone cells to the recovery chamber and allow to rest at the current temperature of the water bath for at least 30 min before single-cell RNA collection (i.e., it is not necessary to increase the temperature of the solution when it has already started to cool down).

⚠ CRITICAL: When cells of interest are found on both surfaces of a slice (Figure 2), recover with the side containing the most number of cells facing up to maximize survival.

⚠ CRITICAL: Return slices containing cells to the same well where they were collected into during sectioning in order to keep the same numbering system. The number of discarded slice should be noted to avoid confusion. When multiple brains are collected and screened in parallel, it is recommended to keep each brain separately in different recovery chambers.

Visually-guided single-cell RNA collection

Timing: 1–4 h

This section describes the procedures of collecting the content of individual clonally-related cells for RNA sequencing. Acute slices are best used within 4–6 h after preparation and should not be kept longer than 6 h. It is thus advisable to maximize this time to achieve as much clonal coverage/sampling as possible.

11. Perform the following steps to minimize potential RNase contamination:
    a. Clean electrophysiology setup, pipette puller and all work surfaces using RNase away.
    b. Put on a lab coat and gloves when handling samples.
    c. Leave all components which will come into direct contact with the collected samples covered from air and dust.
12. Prepare fresh pipette internal solution:
    a. Make 300–400 μL of 1× RNase free PBS solution containing 0.4% RNase inhibitor.
    b. Filter internal solution using 0.22 μm filter into a fresh RNase free Eppendorf.
    c. Keep on ice and use within the day of preparation.
13. Prepare glass pipettes for sample collection:
    a. Using pipette puller, pull glass pipettes with opening size of about 3–5 μm.

    Note: The pipette puller program consisted of a five-step pull, with the first step producing a relatively long taper (~4–5 mm) due to high pull velocity. The fifth step is used to fine-tune the pipette opening via temperature control. See "materials and equipment" section for an example of pull program to use as a starting point. To generate ideal pipette opening size, it is recommended to inspect a few pre-pulled pipettes at the beginning of every day of experiment and adjust the final temperature and velocity accordingly to manufacturer manual (https://www.sutter.com/manuals/P-1000_OpMan.pdf) to achieve an idea size.

    b. Fill each glass pipette with 3 μL of internal solution using microloader.
    c. Remove any air bubbles at the tip of the glass pipette by gentle tapping.

    ⚠ CRITICAL: Do not prepare pipettes too early in advance and be sure to cover all prepared pipettes from dust.

14. Transfer single acute slice in electrophysiology setup with constant perfusion of carbogenated 1× ACSF.
15. Locate cells to be collected first using a low magnification (4× or 20×) and then a high magnification (63×) objectives.

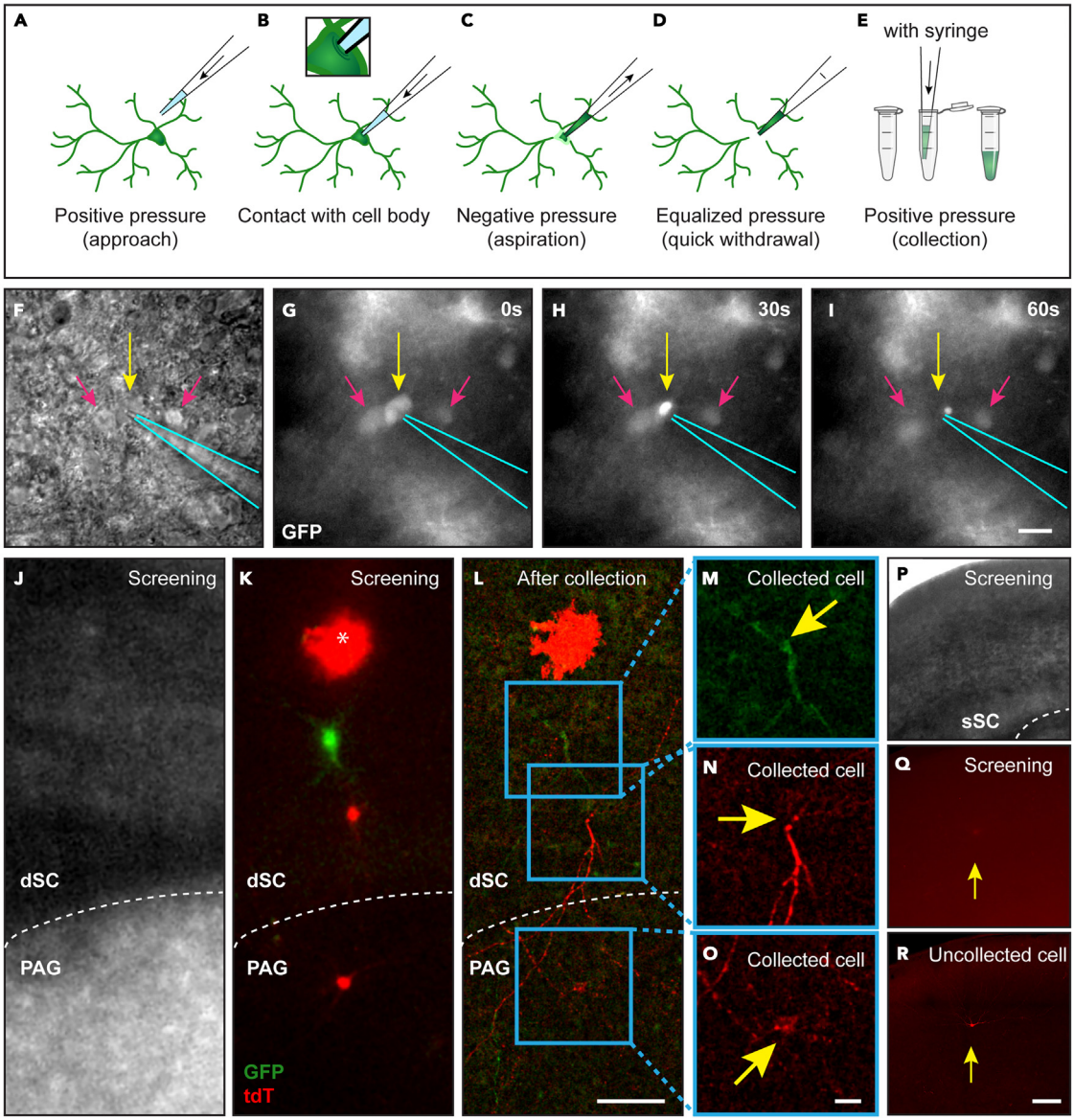


Figure 3. Visually-guided single-cell RNA collection

(A–E) Schematics illustrating the steps of RNA collection from a single fluorescent cell in acute brain slices.

(F–I) Images illustrating RNA collection from a single GFP positive cell (yellow arrow) while two neighboring cells (pink arrows) remain undisturbed. Glass pipette is outlined in blue.

(J–O) Images showing verification of 3 collected neurons. Bright field (J) and fluorescence (K) images obtained during screening stage showing 1 green (GFP) and 2 red (tdT) neurons and a red astrocyte (marked by white asterisk). Post-collection assessment with confocal imaging (L) with higher magnification images shown in (M–O). Yellow arrows point to collected cell bodies.

(P–R) Images showing a tdT-positive cell (yellow arrow) which were not collected due to its deep position in the brain slice. This cell was barely visible during pre-screening (P–Q) but revealed after confocal imaging (R). Layers are separated by lines and marked as superficial superior colliculus (sSC), deep superior colliculus (dSC) and periaqueductal gray (PAG). Scale bars = 10 μm in (F–I and M–O) and 50 μm in (J–L and P–R).

16. Mount filled pipette on pipette holder.
17. Insert the pipette into the ACSF solution in the perfusion chamber directly above the cell of interest.
18. Insert the glass pipette into the acute slice while applying a slight positive pressure through the pipette to prevent clogging or contamination with other cellular material (Figure 3A).

Note: To apply pressure into the pipette, connect the pipette holder with a syringe via a clean tubing. Troubleshooting problem 4.

19. Using a 63× objective, approach the cell of interest under bright field view. Verify the location of the cell with fluorescence briefly if necessary.

Note: Do not use strong fluorescence intensity and long exposure to minimize bleaching of the cell of interest.

20. Position the pipette opening directly on top of and touching the cell body. A small indentation can be observed at the point of contact (Figure 3B).
21. Release positive pressure and switch to fluorescence view.
22. Apply gentle and constant negative pressure to aspirate the cell body into the pipette (Figure 3C). A successful collection should take about 20 and not more than 60 s (Figures 3F–3I; Methods video S1).

Note: At this point, a distortion of the cell body should be observed followed by an observable flow of fluorescent materials into the pipette. Troubleshooting problem 5.

23. After collection of the cell body, immediately release negative pressure to avoid collecting unwanted material (Figure 3D). Quickly remove pipette from the slice. Troubleshooting problem 6.

Note: We find that removing the pipette by pulling it out of the slice diagonally to its surface helps with preventing contaminating materials from attaching to the outside of the pipette during the retrieval process.

24. Insert the tip of the pipette at the bottom of a clean PCR tube (Figure 3E).
25. Carefully connect the top of the pipette with a thin tubing connected to a syringe. This should be done while keeping the tip of the pipette within the PCR tube but without breaking the pipette tip.
26. Simultaneously press the syringe while gently breaking a small tip off the pipette to allow a bigger opening for complete ejection of the content of the pipette into the PCR tube.
27. Immediately close the lid of the PCR tube and spin down briefly on a bench top centrifuge.
28. Transfer tube on dry ice.
29. For documentation, note the color and location of the cell and any additional remarks specific to sample collection.

Note: The alternating dense cell body and fibrous layers of the superior colliculus observable at low magnification under the microscopy allows easy identification of layer location of cells either in the superficial (sSC), deep (dSC) layers or the periaqueductal gray (PAG). 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). It is also helpful to document the position of each cell collected by taking a low magnification image to show the position of the tip of the pipette where the cell was located.

30. Continue to collect from the next cell of the clone in the same manner. We routinely collect around 20 MADM clone cells in one session (about 3–4 h) of single-cell RNA collection.

⚠ CRITICAL: When multiple cells are to be collected in the same slice, it is important to first decide on the order of collection to avoid damaging subsequent cells to be collected. We suggest to start from the most superficial to deepest location in terms of the depth of the slice. When multiple cells are found at the same depth, always collect from the one closest to the pipette position. In very dense situation, consider rotating the acute slice in the perfusion chamber and try to approach a dense cluster from different directions outside in.

31. Transfer samples in PCR tubes to −80°C at the end of a day of sample collection.

Note: It is useful to collect a few negative control samples containing only 4 μL of internal solution for quality control at a later stage (step 38). Alternatively, insert a fresh pipette with the same amount of internal solution in the brain slice for a few minutes without applying negative pressure. This will detect any contaminating material which may enter or adhere to the outside of the pipette.

32. When all cells from one acute slice have been collected, fix slice in 4% PFA at 4°C for 16–24 h in 24 well plates.

MADM clone reconstruction and clonal coverage assessment

Timing: 4 days

This section describes procedures to check for the percentage of sampling of MADM clones and clonal reconstruction.

33. Perform immunostaining of fixed acute slices to amplify GFP and tdT signals and mark cell layers with DAPI:
    a. While keeping fixed acute slices in individual wells of a 24-well plate, wash with 1× PBS three times for 10 min each on a gentle shaker.
    b. Block acute slices using 10%1X PBT and 5% Donkey Serum for 3 h at 22–25°C on shaker.
    c. Incubate slices in primary antibodies, chicken anti-GFP (1:500) and goat anti-tdT (1:500), in blocking solution at 4°C on shaker for 48 h, cover from light.
    d. Wash three times with 1X PBT for 5 min each.
    e. Incubate slices in secondary antibodies, anti-chicken 488 (1:500) and anti-goat 568 (1:500) in 1X PBT at 22–25°C for 2 h on shaker, cover from light.
    f. Wash twice with 1X PBT and once with 1× PBS on shaker for 5 min each.
    g. Incubate slices with DAPI working solution at 22–25°C for 15 min on shaker.
    h. Wash for 5 min with 1× PBS.
    i. Mount slices on glass slides with Mowiol-DABCO solution and cover with cover glass.
34. Inspect stained sections for missed cells using epifluorescence microscope (e.g., Zeiss Axio Imager 2 Microscope). Acquire images using a confocal microscope (e.g., Zeiss LSM 800 confocal microscope) if necessary.

Note: Using pre-screening images obtained before sample collection as reference, collected cells with remaining processes can also be observed and inspected. The steps to amplify GFP and tdT signals are essential to making cellular processes more visible. It is therefore also possible to reconstruct the MADM clone post collection (Figures 3J–3O). The completeness of collection and any unpatched cells (Figures 3P–3R) can then be assessed.

RNA sequencing and cell-type identification of clonally-related cells

Timing: 6–8 weeks

This section describes downstream experimental approaches to determine cell-type identity of individual MADM-CloneSeq cells using RNA sequencing followed by bioinformatics data analyses. While details for each step are beyond the scope of this protocol, we provide a general overview of major experimental steps (Figure 4). We refer readers to our original paper[1] and associated scripts (https://github.com/fpauler/Multipotent-Progenitors-Instruct-Ontogeny-of-the-Superior-Colliculus) for more details on the experimental approaches and analysis pipelines. We also provide references to other resources wherever appropriate.

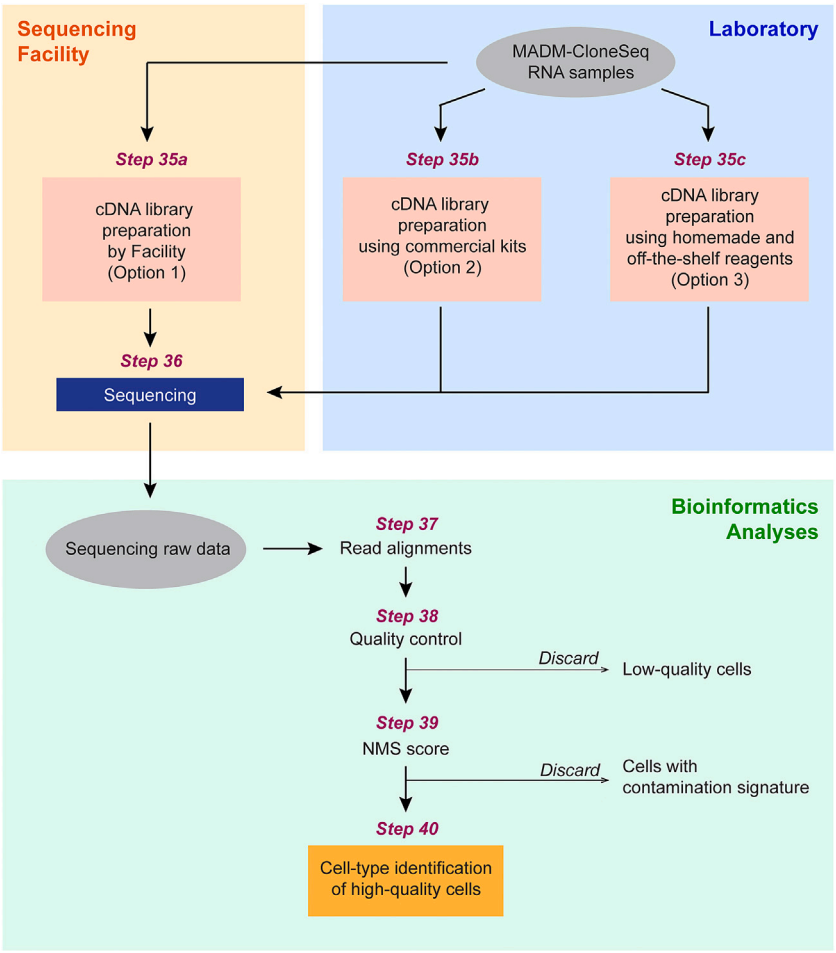


Figure 4. Overview of major experimental steps for RNA sequencing and data analysis

A schematic overview of the major experimental steps including three options for cDNA preparation, RNA sequencing, and data analyses of sequencing data. Steps performed in the laboratory (Blue box), at the sequencing facility (yellow box) and by bioinformatics analyses (green box) are marked. Note that corresponding steps in the protocol are referred to in the figure.

⚠ CRITICAL: A suitable approach for cDNA library preparation (step 35) should be chosen based on the availability of sequencing facilities, as well as financial and other concerns. See "considerations for eventual cDNA library preparation approaches" under "*Before you begin*" section. It is crucial to communicate with relevant sequencing facilities before beginning sample collection to determine specific requirements for sample submission and storage conditions.

35. Generate cDNA libraries from collected MADM-CloneSeq RNA samples:
    a. Depending on the chosen approach for cDNA library preparation shown in *Before you begin* section, go to step 35b for Option 1; 35c for Option 2; or 35d for Option 3.
    b. Submit RNA samples directly to a sequencing facility for cDNA library preparation (Option 1). Go to step 36.

Note: We recommend using Smart-seq v2[10] or v3[11] methods commonly available at sequencing facilities.

c. Use commercially available kits to prepare cDNA libraries in the laboratory from stored RNA samples (Option 2). Go to step 36.

Note: We recommend using SMART-Seq Single Cell Plus kit by following manufacturer's protocol.

d. Perform cDNA library preparation using homemade and off-the-shelf reagents in the laboratory from stored RNA samples (Option 3). Go to step 36.

Note: Several protocols offer possibilities to prepare reagents in the laboratory, which are otherwise available commercially for a higher price. See Picelli et al., 2014,[10] Cadwell et al., 2017[8] and Hagemann-Jensen et al., 2020[11] for detailed components of reagents and protocols.

36. Perform sequencing of prepared libraries at designated sequencing facilities.

Note: The Illumina platform offers several possibilities for sequencing. We recommend the NovaSeq System (https://www.illumina.com/systems/sequencing-platforms/novaseq.html). Alternative sequencing platforms are also emerging and are worth considering for specific needs.[15]

Note: Sequencing should be performed with adequate depth suitable for the research question of interest. In order to ensure that accurate expression profiles of cell-types with complex transcriptomes are obtained, we recommend ~2 million reads per cell which should provide data for >5,000 genes.[16]

37. After obtaining RNA sequencing raw data, perform standard procedures for read alignment to determine gene expression.[17]

Note: Several different gene expression measurements have been proposed[17] and the optimal choice depends on the specific library preparation method as well as on the reference dataset.

38. Filter out low-quality cells based on number and fraction of aligned reads as well as percentage of mitochondria transcripts.

Note: We suggest to include a set of negative controls in the analysis to determine exact cut-offs, i.e. samples with only internal solution or solution from pipettes inserted in the brain slice without active aspiration. In doing so, even partially collected cells could be processed and analyzed as long as the quality measures are above negative control level.

39. Filter out potentially contaminated samples. We found that the Normalized Marker Sum (NMS)[18] provides good measure for the determination of contaminating signatures.

⚠ CRITICAL: This step is crucial to identify potential contamination from RNA of surrounding non-targeted cell types during sample collection. For example, glial cells would be a contaminating type when only neurons were collected. Such contaminating gene expression signatures might negatively impact on downstream analyses.

Note: NMS score is a measure of the expression level of different sets of cell-type specific marker genes extracted in a MADM-CloneSeq cell relative to the reference dataset. Thus, an NMS score reports how transcriptionally similar a collected cell is to the targeted versus a contaminating cell type. Available adult single-cell or single-nuclear RNA sequencing

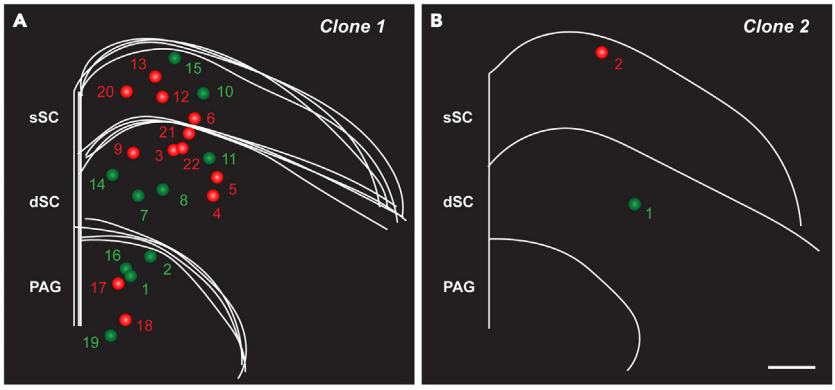
reference datasets of the SC are still growing. Some examples are Zeisel et al., 2018,[19] Xie et al., 2021,[20] Liu et al., 2023,[21] and Choi et al., 2023.[22] Depending on the reference dataset chosen, scaling of the NMS cutoff value might be necessary to correct for differences in absolute gene expression levels between reference and MADM-ClonSeq datasets. Troubleshooting problem 7.

40. Map identity of each cell using existing reference datasets.

Note: MADM-CloneSeq, similar to any other Patch-Seq methods produces noisy data, which makes it necessary to assign cell types based on similarity to a single-cell or single-nuclear RNA sequencing reference dataset.[19–22] Optimal statistical methods to determine similarity depend on library preparation method as well as reference datasets and might need to include batch correction. See our original paper for cell-type assignment used to define SC neuronal types.[1]

EXPECTED OUTCOMES

MADM-CloneSeq takes advantage of combining MADM clonal labeling and RNA sequencing to uncover cell-type identity of sparsely labeled clonally-related cells. Two examples of MADM SC clones with correlated cell-type identity are illustrated in Figure 5. The first clone was induced at embryonic day (E) 10.5 (Figure 5A) whereas the second clone was later at E12.5 (Figure 5B). Both clones were



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Clone 1						Clone 2	
Cell	Cell-type	Cell	Cell-type	Cell	Cell-type	Cell	Cell-type
1	SCINH4	9	SCEXC1	17	SCEXC2	1	SCINH1
2	SCINH1	10	SCINH2	18	SCEXC2	2	SCEXC5
3	SCINH1	11	SCEXC1	19	SCEXC2		
4	SCEXC2	12	SCINH6	20	SCINH8		
5	SCEXC6	13	SCINH8	21	SCINH1		
6	SCEXC5	14	SCINH1	22	SCINH7		
7	SCEXC3	15	SCEXC5				
8	SCINH1	16	SCEXC2				

Figure 5. Representative MADM clones with mapped cell-type identity of individual cells

(A and B) Reconstructed illustrations of 2 MADM clones in the SC are shown. Red and green spheres represent the positions of individual sampled MADM-labeled cells. Layers are separated by lines and marked as superficial superior colliculus (sSC), deep superior colliculus (dSC) and periaqueductal gray (PAG). A Z-projection from 4 acute slices are shown for Clone 1 which was induced at E10.5 and contains 22 neurons (A). Clone 2 was induced at E12.5 and contains 2 neurons in a single slice (B).

(C) Cell-type identity of individual cells is listed for both clones. Inhibitory types are written in blue while excitatory types are in orange. Cell types are annotated based on published dataset[19] and renamed in our original publication.[1]

Scale bars = 200 μm in (A-B).

sampled at postnatal day (P) 28. Both clones provide direct evidence that multiple cell types originate from the same progenitor where MADM events occurred, and thus share the same lineage (Figure 5C). Based on the dual color labeling of MADM, we can also infer closer lineage relationships among cells of each subclone color. The smaller clone has remarkably captured the results of the final division of a progenitor where, in this case, SCIHN1 and SCEXC5 types were generated. Furthermore, we have also retained the spatial information of each cell and obtained information about layer distribution of these cell-types. By sampling a number of clones, any potential pattern of cell-type pairing and clonal composition could be investigated. Thus, the precision of MADM-CloneSeq allows us to determine the outcome of even the final cell division producing only two cells.

Clonal coverage for each clone can be determined by taking into account the number of cells that were missed during sample collection (step 34) and the number of collected cells that did not pass quality control (step 38–39) using the following equation:

$$\textit{Clonal coverage}\ (\%) = \frac{n_{HQ}}{n_{HQ}+n_{LQ}+n_{M}} \times 100$$

where n_{HQ} = the number of high-quality cells that passed quality control,

n_{LQ} = the number of low-quality cells that were filtered out by quality control,

n_{M} = the number of missed cells which were not collected.

It should be emphasized that, due to several technical constraints (see limitations), it is to be expected that clonal coverage would sometimes fall below 100%. Clones with incomplete coverage can still be used as long as conclusions are made from the informative cells only. As a reference, in our original paper,[1] clonal coverage ranged between 30%–100% with the majority of clones reaching 80–100% as shown in the following table.

Clonal coverage (%)	Frequency (%)
30%–40%	14.5%
40%–60%	25.0%
60%–80%	27.6%
80%–100%	32.9%

LIMITATIONS

Two major limitations of MADM-CloneSeq relate to its inherent low throughput and high cost. Low throughput stems from the fact that MADM clones are extremely sparse by nature to allow us to claim clonality. Higher throughput can be achieved by having multiple experimenters working together for designated tasks like preparing glass pipettes, documentations, collecting cells and processing collected RNA. The high cost for MADM-CloneSeq is associated with the cDNA preparation and RNA sequencing steps (steps 35–36). Considerable effort should be invested in initial quality control for RNA quality before proceeding with larger scale experiments for subsequent steps. Since the protocol is designed to correlate between clonal relationship and cell-type identity, it is essential to sample as many cells as possible within a clone. There are two specific limitations that could prevent us from achieving this goal. The first one is the short time window during which acute brain slices remain in good quality. The size of a clone to be sampled should be taken into consideration before beginning to collect. A clone larger than 20 cells may not be collected completely before the slices are getting too old. Another limitation is the location of individual cells which makes access with a glass pipette difficult. Cells that are too deep in the brain slice are less visible and thus difficult to collect compared to one on the surface (Figures 3P–3R). Cells next to or below a blood vessel or axonal tracks are often not easily visible. Given these constraints, it is thus advisable to take these aspects into account when screening for clones and give priority to collecting clones that are of manageable size and depth. When interpreting data from

clones with incomplete coverage, it is important to determine whether sampling of different subregions of the brain (i.e., SC layers) was biased by depth of the slices. See Figure S6H of our original paper[1] showing that the distribution of all informative cells in a clone across layers was similar to the distribution of all cells in the clone. Despite these limitations, MADM-CloneSeq offers unique and extremely precise and highly correlative readout of lineage, morphology, spatial and transcriptomic identities.

TROUBLESHOOTING

Problem 1

Liver of the mouse does not change color during ACSF perfusion.

Potential solution

Reposition the needle in the heart such that the tip of the needle stays within the left ventricle. Be sure that the cut made to the right atrium is large enough to allow ACSF to leave the circulation. Increase perfusion rate moderately if necessary.

Problem 2

Two distinct clusters of cells found in one brain.

Potential solution

We use a 300 μm minimum distance of separation to define separate clones. If two clones are found, priority should be given to the one where cells are closer to the surface to enhance sampling coverage.

Problem 3

No or too many clones per brain.

Potential solution

If there are no clones after screening all slices or it is difficult to define clonality because of too many labeled cells, prepare new acute slices from a different mouse. If this problem persists, consider adjusting the concentration of tamoxifen during clonal induction or using a different CreER-driver.

Problem 4

No solution coming out of the glass pipette when positive pressure is apply.

Potential solution

Pipette might be clogged with debris. With positive pressure, any potential debris in the glass pipette will be pushed to the tip and is sometimes visible. Applying stronger pressure can sometimes help with removing small debris or bubbles. Replace pipette if necessary. If this continues, filter internal solution again.

Problem 5

Aspiration appears to lead to no transfer of material or too rapid.

Potential solution

Check under bright field view, re-adjust the position of the pipette if necessary. It is possible that the cell body has moved upon manipulation. If this continues, check pipette holder and tubing controlling pressure for leak. Replace tubing or pipette holder if necessary. It is sometimes possible for the nucleus to get stuck at the tip and require longer to pass through the opening. If this is the case, wait for 5–10 s for this to happen without applying more suction (Methods video S2). For large cell body, a larger pipette opening can facilitate collection. However, a pipette opening too large may lead to collection of contaminating material (Methods video S3).

Problem 6

Parts of the cell body remain after aspiration.

Potential solution

Depending on the surrounding tissue density and cell morphology, some cell bodies do not become completely aspirated. In this case, it is important to collect partially rather than leave pipette in too long to collect unwanted material. Consider using larger opening of pipettes in the next approach. See Methods video S4.

Problem 7

Absolute gene expression levels between reference and MADM-CloneSeq datasets are of different scales making the interpretation of a relative ratio and thus determining a NMS cutoff value difficult.

Potential solution

This problem could arise when different sequencing methods were used to generate the two datasets. For example, in our dataset from the original paper,[1] TPM gene expression values obtained using Smart-seq method was ~8× higher than UMI counts from a 10× Genomics reference dataset.[19] In this case, the cutoff NMS value was scaled up accordingly by a factor of 8 (from a recommended value[18] of 0.4 to 3) to correct for the unmatched scales. We refer readers to Figure S6E of our original paper[1] for a plot of NMS score showing that, after the scale adjustment, only cells with a neuron NMS score >3 and a contaminating NMS score <4 were retained for further analysis.

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 contacts, Giselle Cheung (giselle.ty.cheung@gmail.com) or Florian M. Pauler (florian.pauler@ist.ac.at).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No dataset or new code was generated while composing this protocol.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103168.

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AUTHOR CONTRIBUTIONS

G.C., F.M.P., 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 interests.

REFERENCES

1. Cheung, G., Pauler, F.M., Koppensteiner, P., Krausgruber, T., Streicher, C., Schrammel, M., Gutmann-Özgen, N., Ivec, A.E., Bock, C., Shigemoto, R., and Hippenmeyer, S. (2024). Multipotent progenitors instruct ontogeny of the superior colliculus. Neuron *112*, 230–246.e11. https://doi.org/10.1016/j.neuron.2023.11.009.

2. Zong, H., Espinosa, J.S., Su, H.H., Muzumdar, M.D., and Luo, L. (2005). Mosaic analysis with double markers in mice. Cell *121*, 479–492. https://doi.org/10.1016/j.cell.2005.02.012.

3. Contreras, X., Amberg, N., Davaatseren, A., Hansen, A.H., Sonntag, J., Andersen, L., Bernthaler, T., Streicher, C., Heger, A., Johnson, R.L., et al. (2021). A genome-wide library of MADM mice for single-cell genetic mosaic analysis. Cell Rep. *35*, 109274. https://doi.org/10.1016/j.celrep.2021.109274.

4. Hippenmeyer, S., Youn, Y.H., Moon, H.M., Miyamichi, K., Zong, H., Wynshaw-Boris, A., and Luo, L. (2010). Genetic mosaic dissection of Lis1 and Ndel1 in neuronal migration. Neuron *68*, 695–709. https://doi.org/10.1016/j.neuron.2010.09.027.

5. Gao, P., Postiglione, M.P., Krieger, T.G., Hernandez, L., Wang, C., Han, Z., Streicher, C., Papusheva, E., Insolera, R., Chugh, K., et al. (2014). Deterministic progenitor behavior and unitary production of neurons in the neocortex. Cell *159*, 775–788. https://doi.org/10.1016/j.cell.2014.10.027.

6. Beattie, R., Streicher, C., Amberg, N., Cheung, G., Contreras, X., Hansen, A.H., and Hippenmeyer, S. (2020). Lineage Tracing and Clonal Analysis in Developing Cerebral Cortex Using Mosaic Analysis with Double Markers (MADM). J. Vis. Exp. *159*, e61147. https://doi.org/10.3791/61147.

7. Hippenmeyer, S. (2023). Principles of neural stem cell lineage progression: Insights from developing cerebral cortex. Curr. Opin. Neurobiol. *79*, 102695. https://doi.org/10.1016/j.conb.2023.102695.

8. Cadwell, C.R., Scala, F., Li, S., Livrizzi, G., Shen, S., Sandberg, R., Jiang, X., and Tolias, A.S. (2017). Multimodal profiling of single-cell morphology, electrophysiology, and gene expression using Patch-seq. Nat. Protoc. *12*, 2531–2553. https://doi.org/10.1038/nprot.2017.120.

9. Amberg, N., and Hippenmeyer, S. (2021). Genetic mosaic dissection of candidate genes in mice using mosaic analysis with double markers. STAR Protoc. *2*, 100939. https://doi.org/10.1016/j.xpro.2021.100939.

10. Picelli, S., Faridani, O.R., Björklund, A.K., Winberg, G., Sagasser, S., and Sandberg, R. (2014). Full-length RNA-seq from single cells using Smart-seq2. Nat. Protoc. *9*, 171–181. https://doi.org/10.1038/nprot.2014.006.

11. Hagemann-Jensen, M., Ziegenhain, C., and Sandberg, R. (2022). Scalable single-cell RNA sequencing from full transcripts with Smart-seq3xpress. Nat. Biotechnol. *40*, 1452–1457. https://doi.org/10.1038/s41587-022-01311-4.

12. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682. https://doi.org/10.1038/nmeth.2019.

13. Ting, J.T., Daigle, T.L., Chen, Q., and Feng, G. (2014). Acute brain slice methods for adult and aging animals: application of targeted patch clamp analysis and optogenetics. Methods Mol. Biol. *1183*, 221–242. https://doi.org/10.1007/978-1-4939-1096-0_14.

14. Bischofberger, J., Engel, D., Li, L., Geiger, J.R.P., and Jonas, P. (2006). Patch-clamp recording from mossy fiber terminals in hippocampal slices. Nat. Protoc. *1*, 2075–2081. https://doi.org/10.1038/nprot.2006.312.

15. Eisenstein, M. (2023). Illumina faces short-read rivals. Nat. Biotechnol. *41*, 3–5. https://doi.org/10.1038/s41587-022-01632-4.

16. Lipovsek, M., Browne, L., and Grubb, M.S. (2020). Protocol for Patch-Seq of Small Interneurons. STAR Protoc. *1*, 100146. https://doi.org/10.1016/j.xpro.2020.100146.

17. Gondane, A., and Itkonen, H.M. (2023). Revealing the History and Mystery of RNA-Seq. Curr. Issues Mol. Biol. *45*, 1860–1874. https://doi.org/10.3390/cimb45030120.

18. Lee, B.R., Budzillo, A., Hadley, K., Miller, J.A., Jarsky, T., Baker, K., Hill, D., Kim, L., Mann, R., Ng, L., et al. (2021). Scaled, high fidelity electrophysiological, morphological, and transcriptomic cell characterization. Elife *10*, e65482. https://doi.org/10.7554/eLife.65482.

19. Zeisel, A., Hochgerner, H., Lonnerberg, P., Johnsson, A., Memic, F., van der Zwan, J., Haring, M., Braun, E., Borm, L.E., La Manno, G., et al. (2018). Molecular Architecture of the Mouse Nervous System. Cell *174*, 999–1014.e22. https://doi.org/10.1016/j.cell.2018.06.021.

20. Xie, Z., Wang, M., Liu, Z., Shang, C., Zhang, C., Sun, L., Gu, H., Ran, G., Pei, Q., Ma, Q., et al. (2021). Transcriptomic encoding of sensorimotor transformation in the midbrain. Elife *10*, e69825. https://doi.org/10.7554/eLife.69825.

21. Liu, Y., Savier, E.L., DePiero, V.J., Chen, C., Schwalbe, D.C., Abraham-Fan, R.J., Chen, H., Campbell, J.N., and Cang, J. (2023). Mapping visual functions onto molecular cell types in the mouse superior colliculus. Neuron *111*, 1876–1886.e5. https://doi.org/10.1016/j.neuron.2023.03.036.

22. Choi, J.S., Ayupe, A.C., Beckedorff, F., Catanuto, P., McCartan, R., Levay, K., and Park, K.K. (2023). Single-nucleus RNA sequencing of developing superior colliculus identifies neuronal diversity and candidate mediators of circuit assembly. Cell Rep. *42*, 113037. https://doi.org/10.1016/j.celrep.2023.113037.