Mosaic Analysis with Double Markers (MADM) in Mice

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot080366

Protocol

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The human brain comprises more than 100 billion neurons, each of which has an elaborate shape and a complex pattern of connections. To untangle this complexity, it is often useful to visualize one neuron at a time. Mosaic analysis with double markers (MADM) is a genetic method for labeling and manipulating individual neurons. This method was developed in mice and it allows simultaneous labeling and gene knockout in clones of somatic cells or isolated single cells in vivo. In MADM, labeling is achieved by using site-specific recombinases to induce the reconstitution of chimeric fluorescent proteins. Here we provide the standard procedure for utilizing MADM to examine lineage analysis, neural circuit tracing, and gene function. *ROSA26*-MADM is used as an example because the reagents are published and available. As MADM cassettes are introduced onto more chromosomes, genes located on these other chromosomes can be subjected to mosaic analysis in an analogous manner to that described below. We present detailed protocols with troubleshooting guides, as well as applications of the technique in tracing neural circuits, live imaging of developing neurons, and studying mechanisms of neuronal morphogenesis.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Antibodies

Chicken anti-green fluorescent protein (anti-GFP) (1:500; Aves Labs GFP-1020)

Goat anti-Myc (1:200; Novus NB600-338)

General immunohistochemistry reagents

Mice for MADM analysis (available at The Jackson Laboratory; MADM-GR stock no. 006075 and MADM-RG stock no. 006080)

Mutant mice (available at The Jackson Laboratory and the scientific community)

The International Knockout Mouse Consortium (IKMC; http://www.knockoutmouse.org) is in the process of mutating all protein-coding genes in the mouse.

Tamoxifen (20 mg/mL in corn oil; Sigma-Aldrich C8267)

Used to induce mitotic recombination if using inducible Cre-ER lines; see Discussion section.

Transgenic mice expressing Cre or the inducible Cre-ER lines

These mice are readily available from The Jackson Laboratory (http://jaxmice.jax.org), the Allen Institute for Brain Science (http://transgenicmouse.alleninstitute.org), GENSAT (http://www.gensat.org/cre.jsp), and the scientific

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080366

community (for a list, see http://nagy.mshri.on.ca/cre_new/index.php). Cre may also be delivered by virus or electroporation methods.

Equipment

Microscope

Imaging of MADM clones can be achieved using a range of microscopes, depending on the tissue of interest, resolution desired, and experimental analysis. In general, a standard confocal microscope and imaging software is sufficient to obtain high-resolution images for most sectioned and stained tissues. Multi-photon microscopy can be used to visualize cell bodies and dendrites of MADM clones in vivo.

PCR machine for genotyping
Peristaltic perfusion pump
Standard mouse room facilities
Tissue sectioning equipment (e.g., cryostat)

METHOD

In the MADM system (Zong et al. 2005), labeling is achieved by recombination-induced reconstitution of chimeric fluorescent proteins (Fig. 1A). These two chimeric proteins are needed to distinguish chromosome segregation types. If the parental cell carries one copy of the normal gene and one copy of the mutant gene (heterozygous for the mutation), only single-colored progeny would alter the genotype—either homozygous for the normal allele or homozygous for the mutant allele (Fig. 1A, G_2-X). Because the progeny are siblings of the same cell division, mutant-induced phenotypes can be studied by directly comparing the two siblings, each with distinct fluorescent markers. Double-labeled cells in MADM derived from a different chromosome segregation type (Fig. 1A, G_2-Z) or from recombination in G_1 or postmitotic cells (Fig. 1A, G_1/G_0) do not alter the genotypes.

Cross Cre-Expressing Mice with MADM-RG Mice

1. Obtain the following two mouse lines: mice carrying the *GR* cassette (MADM-GR) and mice carrying the reciprocal *RG* cassette (MADM-RG).
2. Generate or obtain a tissue-specific or ubiquitous Cre driver mouse line or a temporally inducible Cre-ER driver mouse line and cross with MADM-RG mice (Fig. 2A).

   Mice carrying both a MADM cassette and Cre driver may be weak from the presence of multiple genetic elements. The choice of Cre driver line depends on experimental analysis. Cre expression in mitotic cells is necessary for mutant analysis. The efficiency of clone generation depends on the genetic components used and the tissue being analyzed.

   See Troubleshooting.

Recombine Mutation Distal to the *GR* Cassette and Generate MADM–Green–KO Clones

3. Introduce a mutation onto the chromosome arm distal to the *GR* cassette located in the *ROSA26* locus on chromosome 6. To recombine an existing mutation, use the following two simple crosses.

   i. Cross mice heterozygous for an existing mutation distal to the *ROSA26* locus with MADM-GR mice.

   ii. Cross trans-heterozygous progeny with mice carrying the *RG* cassette and *Cre* transgene (Fig. 2). The mutation is recombined distal to the *GR* cassette following standard meiotic recombination. See Discussion for considerations.

   In these mutant MADM mice, Cre also drives Cre/loxP-directed mitotic recombination to generate fluorescently labeled homozygous mutant, homozygous wild-type, and heterozygous mutant cells. Green fluorescent homozygous mutant and red fluorescent wild-type cells are generated when the mutation is introduced distal to the GR *cassette (e.g., Fig. 2A). Red fluorescent homozygous mutant and green fluorescent wild-type cells are generated when the mutation is introduced distal to the* RG *cassette (e.g., Fig. 2B).*

   See Troubleshooting.

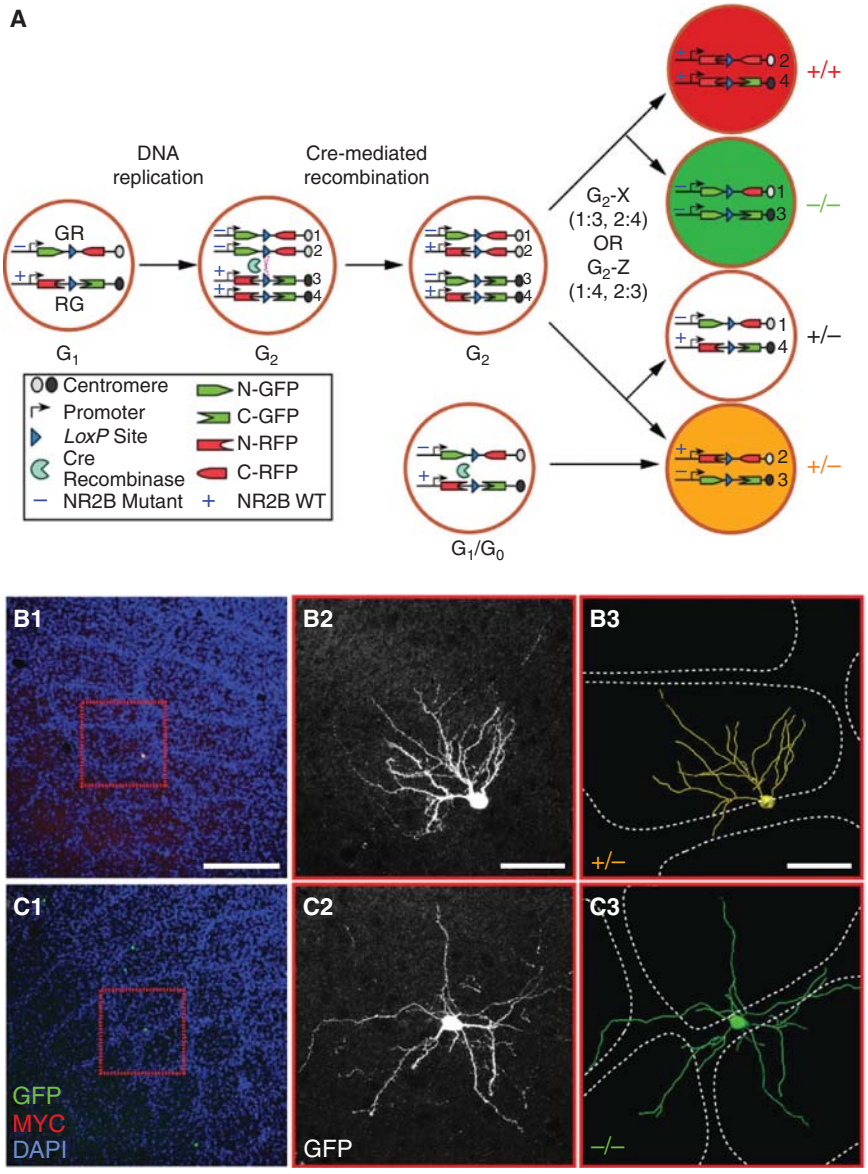

FIGURE 1. Principle and application of the MADM system in mice. (*A*) Schematic of the MADM method illustrating Cre-mediated interchromosomal recombination that results in reconstitution of two fluorescent markers, GFP and red fluorescent protein (RFP) (dsRed2-Myc). If recombination occurs during the G_2 phase, chromatids can segregate to generate two differentially labeled cells with altered genotypes (G_2-X segregation) or one doubly labeled cell and one unlabeled cell without changing the genotype (G_2-Z segregation). Recombination in G_1 or postmitotic cells (G_0) generates a doubly labeled cell without changing the genotype. (*B,C*) Dendrite targeting of layer 4 spiny stellate cells in the barrel cortex (bSCs) of postnatal day 21 mice. (B_1,C_1) Representative low-magnification images show barrels outlined by surrounding dense nuclei staining (DAPI in blue), and sparse MADM-labeled *NR2B*$^{+/-}$ (B_1) and *NR2B*$^{-/-}$ (C_1) bSCs. (B_2,C_2) High-magnification confocal images of *NR2B*$^{+/-}$ (B_2) and *NR2B*$^{-/-}$ (C_2) bSCs (same cell as in red box in B_1 and C_1). (B_3,C_3) 3D reconstructions of bSCs in B_2 and C_2 superimposed over barrel walls representing the edge of barrels (dashed white lines). Scale bars, (B_1,C_1) 200 µm; 50 µm for the rest. (*A*, Reprinted, with permission, from Espinosa and Luo [2008]; *B,C*, reprinted, with permission of Elsevier, from Espinosa et al. [2009].)

Dissect and Stain Tissues

4. Analyze the clones in live or fixed tissues. For protocols for mouse perfusion, brain dissection, and immunostaining of fixed tissues, see Espinosa and Luo (2008). To visualize red fluorescent protein (RFP), use preabsorbed goat anti-Myc. To visualize fine neuronal structures (e.g., dendritic spines or axonal boutons), amplify the GFP signal with chicken or rabbit anti-GFP antibody.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080366

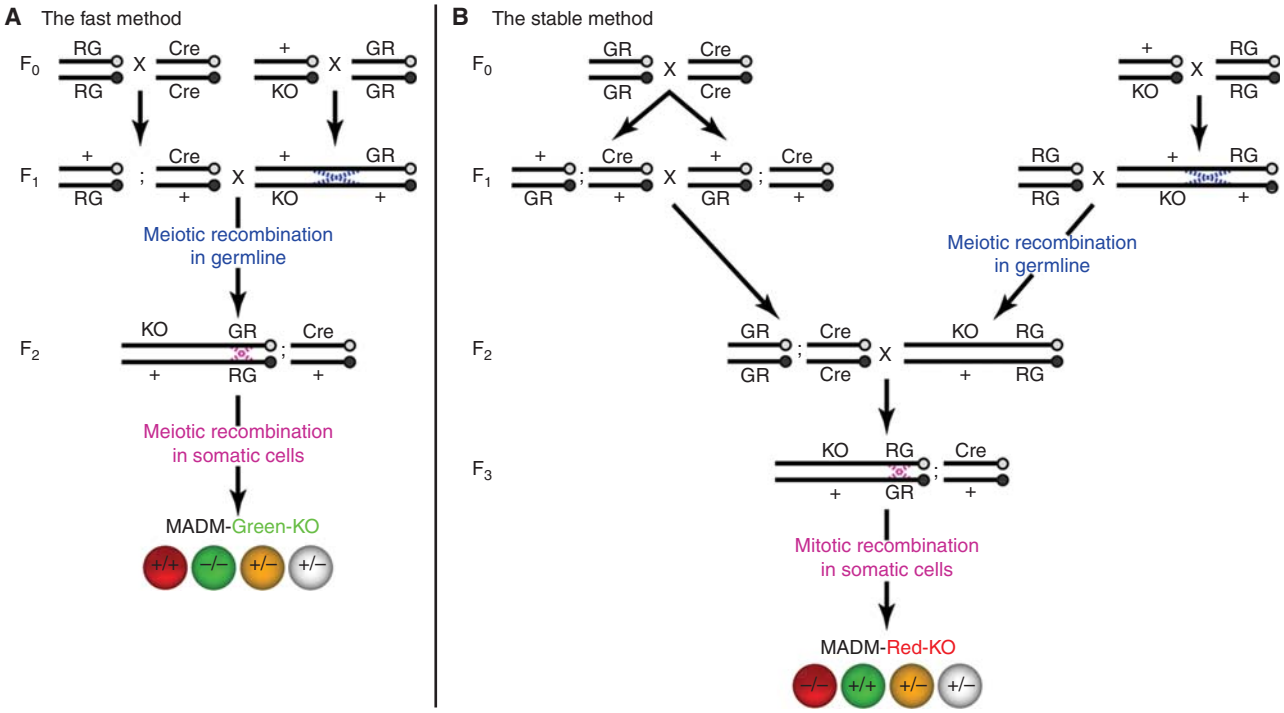

FIGURE 2. Schematic of MADM crossing. (*A*) Fast method of generating MADM mutant mice. This crossing scheme is optimal when the rate of meiotic recombination between the mutation and the MADM cassette is high. Mice carrying the *RG* cassette are crossed to mice expressing a *Cre* transgene of choice. Mice carrying the *GR* cassette are crossed to mice heterozygous for a mutation. Trans-heterozygous progeny are then crossed to mice carrying the reciprocal MADM cassette (*RG*) and *Cre* transgene. Mice that inherit *RG* and *Cre* components and a chromosome that has undergone meiotic recombination between *GR* and the mutant allele can produce green fluorescent-labeled mutant cells (MADM–Green–KO) through *Cre/loxP*-directed mitotic recombination in somatic cells. MADM–Red–KO can be easily generated by crossing mice heterozygous for a mutation with mice carrying the *RG* cassette. (*B*) Stable method of generating MADM mutant mice. This crossing scheme is optimal when the rate of meiotic recombination between the mutation and the MADM cassette is low, and/or a higher percentage of progeny that can generate MADM clones is essential. This scheme requires one additional generation compared to (*A*). Mice carrying the *GR* cassette are crossed to mice expressing a *Cre* transgene of choice. Progeny are intercrossed to generate mice homozygous for the *GR* cassette and *Cre* transgene. Mice carrying the *RG* cassette are crossed to mice heterozygous for a mutation. Trans-heterozygous progeny are then crossed to mice homozygous for the *RG* cassette. Mice can inherit a chromosome that has undergone meiotic recombination between *RG* and the mutant allele. Mutant mice carrying the *RG* cassette are crossed with mice homozygous for the *GR* cassette and *Cre* transgenes. Progeny that inherit *RG*, *GR*, *Cre*, and the mutation produce red fluorescently labeled mutant cells (MADM–Red–KO) through *Cre/loxP*-directed mitotic recombination in somatic cells. MADM–Green–KO can be generated by crossing mice heterozygous for a mutation with mice carrying the *GR* cassette. Note that the F_2 progeny are established stable lines for producing mice for MADM analysis.

Image Fluorescent Cells

5. Image the MADM clones as soon as possible using an appropriate microscope.
   See Troubleshooting.
6. Store the samples in a dark slide holder for up to several months at 4°C or up to several years at −20°C or −80°C.

TROUBLESHOOTING

Problem (Step 2): MADM mice are unhealthy.

Solution: Homozygous alleles, especially homozygous for *GR*, or multiple genetic elements can make mice weak. Maintain some mice heterozygous for the allele, minimize breeding littermates, or amplify mice to compensate for loss.

Problem (Step 3): Few MADM mutant mice survive.

Solution: Low survival rate can be explained by the mutation causing a severe developmental phenotype or the heterozygous mutation having a phenotype. Try using a less-efficient Cre line, amplifying mice to compensate for loss, observing MADM mutant mice lacking Cre, or verifying that the cross does not generate homozygous mutant mice.

Problem (Step 3): The efficiency of clone generation is too low or too high.

Solution: Possible causes include inherent bias of mitotic recombination, Cre expression level, or the mutation studied is causing cell death or reduced proliferation. Use a stronger or weaker Cre driver line, or make the Cre driver line homozygous for stronger or heterozygous for weaker expression levels. If using an inducible Cre-ER driver line, optimize the timing, frequency, dose, or method (intraperitoneal or gavage) of tamoxifen administration. If studying a gene mutation, compare the number of homozygous mutant versus wild-type cells.

Problem (Step 3): Clone generation is heavily biased toward double-labeled (yellow) cells.

Solution: Possible causes include inherent bias of mitotic recombination or Cre expression that is very low or nonexistent during the G_2 phase of the cell cycle. Use a stronger Cre driver line or make existing Cre driver line homozygous. Another approach is to use a Cre driver line that preferentially expresses in mitotic cells (expression earlier in development).

Problem (Step 3): Tamoxifen administration causes few mice to develop.

Solution: Tamoxifen administration may result in mouse toxicity or miscarriage. Dissolve the tamoxifen in corn oil instead of dimethylsulfoxide (DMSO). Consider obtaining high-expressing Cre-ER^{T2} lines that require lower tamoxifen doses. Also, optimize the timing (avoid neonates), decrease the frequency or dose, or change the method (intraperitoneal or gavage) of tamoxifen administration.

Problem (Step 5): Unexpected or no MADM phenotype for a mutant with a known phenotype.

Solution: There are several possible causes: Time of clone generation is before or after the gene function required; gene function varies with cell type; differences between cell-autonomous and non-cell-autonomous phenotypes; heterozygous background mutation causes a non-cell-autonomous phenotype; gene functions earlier in the cell's development than previously studied; differences in assay (in vitro vs. in vivo); the mutation is lost because of meiotic recombination. The following solutions should be considered: Use the inducible Cre-ER driver line to induce clones at an earlier or later stage in development; dissect and analyze tissues in a time course; use Cre drivers that label different classes of cells; to compare cell-autonomous and non-cell-autonomous phenotypes, use weaker and stronger Cre drivers to generate sparser and denser labeling, respectively; observe the presence or absence of phenotypes in homozygous mutant, wild-type, and heterozygous mutant cells across labeling densities; verify that heterozygous cells have no phenotype by comparing MADM double-labeled cells in a heterozygous mutant mouse with cells in a wild-type mouse; rescue gene function during the cell's early development or use alternative methods for postmitotic gene knockout (e.g., "SLICK"); replicate previously used assay(s); verify that progeny have null mutation via genotyping.

Problem (Step 5): MADM phenotype has low penetrance or is variable.

Solution: Possible causes include the gene is required at a very specific time during development, gene function varies with cell type, or the gene is required non-cell-autonomously. The following solutions should be considered: Time dissections and analysis of tissues or, if using an inducible Cre-ER driver line, administer tamoxifen with more temporal precision; use an alternative Cre driver that labels only specific cells in which the gene function is required; and use a weaker Cre

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080366

driver to generate sparser labeling and observe the presence or absence of phenotype in homozygous mutant cells.

Problem (Step 5): The level of marker expression is low.

Solution: Low-level marker expression may result from visualizing cells shortly after recombination or a suboptimal staining protocol. Visualize the cells at least 2 d after recombination. For better visualization of RFP, use anti-Myc antibody preabsorbed for >2 d in wild-type brains or use the avidin–biotin complex (ABC) amplification staining method.

DISCUSSION

Issues to Consider When Generating Mutant MADM Mice

In Step 3 of the above protocol, the frequency of meiotic recombination depends on the distance between the mutation and the MADM cassette so that the probability of meiotic recombination decreases for shorter distances. Indeed, the frequency of meiotic recombination can be estimated from the genetic distance between the MADM cassette and the gene of interest. Depending on the frequency of meiotic recombination, different crossing schemes should be used (Fig. 2, cf. A with B). Genotyping of MADM gene knockout progeny should always be conducted to ensure that the mutation was not lost because of meiotic recombination. Crosses that will generate homozygous gene knockout mice should be avoided. When choosing a gene mutation, it is preferable that the gene plays a cell-autonomous role (e.g., receptors). Genes that are "floxed" (flanked by *loxP* sites) can be used with MADM following *Cre/loxP*-directed germline deletion. When using a tissue-specific or ubiquitous Cre driver line, allow the progeny to develop to the desired stage. The promoter used to drive Cre expression will determine when and where the MADM clones will be present. Verify that Cre expression labels cells of interest before the developmental time of analysis. Generation of mutant cells requires Cre expression during mitosis. When using a temporally inducible Cre-ER driver line, administer tamoxifen dissolved in corn oil (20 mg/mL) at the desired time and dose to induce MADM clones and allow the progeny to develop to the desired stage. Cre recombinase activity peaks within 24 h and subsides 36–48 h after tamoxifen administration (Hayashi and McMahon 2002). The timing of tamoxifen administration in combination with the promoter used to drive Cre-ER expression will determine when and where the MADM clones will be present. Tamoxifen should be administered at a dose and time that will obtain the desired labeling density for the tissue and cell type of interest. To reduce tamoxifen toxicity and the risk of miscarriage in pregnant females, obtain high-expressing Cre-ER^{T2} lines that require lower tamoxifen doses. Generation of mutant cells requires induction of Cre-ER during mitosis.

Applications of MADM

Labeling single neurons in their native environment with genetic methods such as MADM can be useful for three main purposes.

- *Tracing the axon projection and dendrite elaboration patterns of individual neurons.* It is possible to characterize different neuronal types and their connection patterns within a specific brain region or between different regions, to discern information flow and the logic of neural circuit organization. Although the Golgi staining method (Ramón y Cajal 1911) is quite powerful, complete axonal arbors are difficult to recover with this technique. MADM allows for complete reconstruction of axonal arbors, selection of the types of neurons that are singly labeled, and enables the tracing of a neuron's lineage along with the visualization of its projections.
- *Studying molecular mechanisms of dendritic and axonal development and plasticity with high anatomical resolution.* To study the intrinsic and extrinsic regulators of neuronal morphogenesis, it is useful to study the consequences of genetic manipulation with single-neuron resolution. MADM

affords the possibility of disrupting endogenous genes only in labeled and isolated neurons, thus allowing analysis of specific functions of pleiotropic genes.

- *Studying the physiological functions of identified neurons in brain slices or in vivo.* Single neurons in wild-type or mutant animals labeled with vital dyes such as GFP can be used for electrophysiological recording or optical imaging in brain slices or in intact animals. These methods allow for characterization of neural circuit function in wild-type animals and genetic analysis of neural circuit development and function using mutants. It is also possible to label single neurons with other markers, such as genetically encoded Ca^{2+} or voltage indicators or effectors that can alter neuronal activity (Luo et al. 2008), so that physiological properties of uniquely identifiable wild-type or mutant neurons can be assayed and perturbed in behaving animals using state-of-the-art imaging techniques.

MADM has been used to study neuronal activity in dendrite patterning in the mouse brain. For example, *N*-methyl-D-aspartate receptors (NMDARs) play important roles in neural development. NR2B is the predominant NR2 subunit of the NMDAR in the developing brain. MADM knockout of NR2B in isolated single cells revealed its cell-autonomous function in dendrite development. For example, spiny stellate cells in layer 4 of the barrel cortex normally restrict dendrite growth to a single barrel (see Fig. 1B). Loss of NR2B results in stellate cells that maintain dendrites in multiple barrels (see Fig. 1C), without affecting dendritic growth rates, total length, or branch numbers (Espinosa et al. 2009). Thus, NR2B functions cell-autonomously to regulate dendrite patterning to ensure that sensory information is properly represented in the cortex.

Advantages and Limitations

Genetic methods for single-neuron labeling such as MADM have several advantages over traditional Golgi staining. The Golgi method labels random neurons, whereas genetic methods can use defined promoter elements to drive marker expression in neurons confined to a particular class. This can be quite important in tracing neural circuits and in studying genes that affect neuronal morphogenesis. If a particular gene affects dendritic morphogenesis or axon projections, these processes can no longer be used as independent criteria in defining neuronal types. In addition, genetic methods afford the possibility of labeling living neurons, allowing time-lapse studies of the dynamic behavior of neurons in their native environment. Finally, the more complete axonal filling achieved by genetic methods is also a major advantage over the Golgi method. However, genetic methods are limited by the availability of defined promoter elements and existing technology. Traditional methods, such as Golgi staining or intracellular dye fill, will still be very useful for many years to come for their capability to label any neuronal class.

Highly specific promoters allow robust and invariant labeling of the same neurons from animal to animal, and thus are excellent choices for studying the molecular mechanisms of neuronal morphogenesis. Because it is extremely rare to isolate promoter elements that allow single-neuron labeling in a defined brain area, this method is significantly limited by the availability of such special promoters. By the same token, chance insertions of transgenes that happen to be stable and label a very small subset of neurons could also be a very useful tool for studying that particular class of neurons. However, it may take a lot of trial and error, without any guarantees of success, to generate transgenic animals that sparsely label a particular class of neurons.

The MADM strategy couples cell division with the generation of labeled neurons, making it an excellent lineage-tracing tool. In addition, MADM permits genetic manipulations of singly labeled neurons. However, this mitotic recombination-based method can be used only to perturb gene function at the time a neuron is born. In many cases, it is useful to eliminate gene function later on (e.g., to study the physiological function of the gene of interest in the mature brain). The "SLICK" method, which is based on chance insertion of a transgene that expresses the tamoxifen-inducible Cre recombinase as well as a reporter resulting in coexpression of both in a small population of neurons, can be used to inactivate genes with "floxed" alleles in small populations of postmitotic neurons (Young et al. 2008).

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080366

ACKNOWLEDGMENTS

We thank all members of the Luo Laboratory over the past 12 years, whose work has contributed much to the data and discussions in this paper. We are also grateful for critical comments from Weizhe Hong. Research in our laboratory has been supported by funds from the National Institutes of Health, the McKnight Endowment Fund, the Human Frontiers Science Program, and the Howard Hughes Medical Institute.

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