A Method for Lineage Tracing of Corneal Cells Using Multi-color Fluorescent Reporter Mice

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Abstract

Lineage tracing experiments define the origin, fate and behavior of cells in a specific tissue or organism. This technique has been successfully applied for many decades, revealing seminal findings in developmental biology. More recently, it was adopted by stem cell biologists to identify and track different stem cell populations with minimal experimental intervention. The recent developments in mouse genetics, the availability of a large number of mouse strains, and the advancements in fluorescent microscopy allow the straightforward design of powerful lineage tracing systems for various tissues with basic expertise, using commercially available tools. We have recently taken advantage of this powerful methodology to explore the origin and fate of stem cells at the ocular surface using R26R-Confetti mouse. This model offers a multi-color genetic system, for the expression of 4 fluorescent genes in a random manner. Here we describe the principles of this methodology and provide an adaptable protocol for designing lineage tracing experiments; specifically for the corneal epithelium as well as for other tissues.

Video Link

The video component of this article can be found at http://www.jove.com/video/53370/

Introduction

During embryonic development, tissue and organ morphogenesis take place through a precise program that can be described in terms of cell proliferation, cell migration and lineage specification1-4. These biological processes are also involved in maintaining adult tissue homeostasis, which requires stem cell regeneration. Lineage tracing, the identification and tracking of the progeny of a specific type of cell population, provides an important tool for studying embryogenesis and stem cell homeostasis. Indeed, it is increasingly being used in many fields of research. Particularly, lineage tracing became an essential tool in identifying the origin and fate of stem cells in homeostasis and cancer development. This is because it may provide essential information about how the cell behaves in the context of the intact tissue or organism, with minimal experimental intervention, in contrast to cell isolation and in vitro culture or transplantation that may result in unwanted bias.

Traditionally, dyes such as lipid-soluble carbocyanine, which incorporate into the plasma membrane of cells, were used for lineage tracing. Although these types of experiments have successfully led to major discoveries and shaped seminal concepts in developmental biology5-7, they have some limitations, including the difficulty to control the specificity of targeted cells, the unwanted impact of the dye on cell behavior and the dilution of the label over time. Nucleotide analogue labeling approaches have enabled documenting the existence of slow-cycling "label retaining cells" that presumably correspond to quiescent stem cells8-11. However, while this technique could demonstrate the presence of slow cycling cells based on proliferation kinetics over a limited time period it could not provide substantial insights regarding the functionality of stem cells. An optimal lineage tracing methodology should minimize the effect of labeling on the properties of the marked cell, its progeny, or its neighbors. Additionally, it would be beneficial to have control on the labeling induction, namely, to have the ability to tag the cell population of interest at a stage and time dependent manner as well as in a single cell (clonal) manner. A stable and irreversible labeling method that is passed on to all progeny of the founder cell is important so that the label would be retained over time, and not transferred to neighboring cells.

More recently, genetic approaches of cell labeling were developed. In these systems, cell specific promoters can be used to trigger Cre recombinase expression in order to remove a loxP-flanked roadblock and allow the expression of reporter genes such as green fluorescent protein or B-galactosidase reporter genes12,13. This approach enables not only the tracking of cells and their progeny but also permits cell isolation and molecular characterization. Cell tracking at the single cell level became possible by induction of the expression of a single fluorescent reporter gene. One important limitation of this system is the fact that only when very low percentage of cells is labeled it is possible to separate and trace individual clones. To overcome this limitation multicolor reporters can be used. When using multicolor labeling, the tracking of differential fate of neighboring cells in the same niche can be achieved efficiently14-17. Recently, a variety of Brainbow (Br) alleles were developed...
for lineage tracing experiments, enabling a stochastic expression of multiple fluorescent reporter genes utilizing the Cre/lox system. The Cre/lox system consists of the Cre recombinase enzyme, which recognizes and binds to specific DNA sequences such as loxP sites. Following the binding of Cre recombinase, site specific recombination occurs, which causes removal of loxP flanked sequences resulting in random expression of different fluorescent proteins (the mechanism is described below). A variety of Br lines are available for use (see reviews\textsuperscript{16,18,19}). The major differences between the Br strains include (i) differences in the number of fluorescent genes included in the cassette, ranging from 2 (Br 2.0), 3 (Br 1.0) to 4 (Br 1.1, Br 2.1) fluorescent genes, (ii) the presence of reciprocally orientated lox sites to allow gene inversion (Br 2.0-2.1), (iii) the type of lox sites used, and (iv) the expression or silence of fluorescent gene expression prior to Cre activation. The recently established Br 3 offers an improved method for multicolor imaging of neurons. One of the main features of this transcript is that it contains a new set of photostable farnesylated fluorescent proteins, which enable an even staining of the finest neuronal processes\textsuperscript{20}.

Importantly, different versions of Br cassettes may contain the neuronal specific Thy1 promoter or the ubiquitously expressed CAG (CMV) promoter. Finally, while some of the Br transgenic mice may contain a single Br transgene, others may consist of multiple transgene copies, thereby allowing the production of an immense number of possibilities for color combinations to be expressed in each cell (for example see \textsuperscript{16}).

In our study, we used the R26R-Confetti mouse which contains the Br 2.1 cassette\textsuperscript{21}. Br 2.1 is uniquely designed to allow Cre-mediated DNA inversions and not only excisions because of the reciprocal orientation of the loxP sites (Figure 1). Thus, these inversion/excision events which lead to color change can continue as long as Cre is present\textsuperscript{16}. For that reason, an inducible temporal Cre expression system is essential for reliable cell tracking using Br 2.1. As shown in Figure 1, the Br 2.1 contains a ubiquitous CAG promoter followed by loxP-flanked Neo\textsuperscript{\textregistered} cassette, which acts as a transcriptional roadblock, that is followed by 4 fluorescent reporter genes, encoding for green (GFP), yellow (YFP), red (RFP) and cyan (CFP) fluorescent proteins, positioned in two tandems. No fluorescence is expressed prior to Cre activation. Induction of Cre recombinase causes the removal of the Neo-R cassette enabling the random expression of one of the 4 fluorescent proteins in a given cell. The first segment contains loxP-flanked GFP and a reversed YFP, while the second segment contains loxP-flanked RFP and a reversed CFP. These DNA segments may continuously be inverted (using loxP that is in reversed orientation), or excised, as long as Cre recombinase is present. Therefore, a transient and controlled Cre transgene should be used. The Br 2.1 cassette provides an excellent system for distinguishing between overlapping emissions upon signal location: the expression of YFP and RFP is cytoplasmic, the GFP is nuclear and the CFP is bound to the cell membrane. GFP and RFP emissions are easily separated by conventional fluorescent microscopy. In order to separate YFP/GFP/CFP emissions, a spectral confocal imaging system is needed. Altogether, the Br 2.1 cassette allows the random, inducible, and tissue specific expression of one out of four distinct fluorescent genes in each targeted cell. In fact, when a larger number of color combinations is needed, one can generate homozygous mice that contain 2 alleles of Br 2.1, thus resulting in the expression of 2 color tags for each cell and in raising the number of possible color combinations to 10\textsuperscript{22}. Some of the Br mouse strains enable the expression of a much greater number of possible color combinations since multiple copies of the cassette were integrated into their genome\textsuperscript{16}. Yet, in most cases, the four color combinations provided by a single Br 2.1 allele are sufficient. Following the protocol provided here, one can establish this method using a relatively simple setup of spectral microscopy with little if any need for calibration.

Here we provide an adaptable protocol for the use of the R26R-Confetti mice that contain a single Br 2.1 allele, for lineage tracing experiments of the corneal epithelium. This transgenic mouse strain has been used for studying the origin and fate of colon\textsuperscript{21,23} and corneal epithelial stem cells\textsuperscript{22,24}, the presence of stem cell activity in intestinal cancer\textsuperscript{25}, and for characterizing kidney podocyte in focal segmental glomerulosclerosis (FSGS)\textsuperscript{17}.

### Protocol

Ethics Statement: In this study animal care and use were conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the local ethical committee.

1. **Choose a Suitable Cre-recombinase Expressing Mouse Strain**

   1. Out of the great variety of Cre transgenic lines available for purchase\textsuperscript{26} and depending on the targeted tissue to be investigated, choose a Cre transgenic mouse strain in which the Cre recombinase gene is controlled by a promoter that is specific for the tissue of interest. In case temporal control is needed, choose an inducible Cre line. Note: We chose the tamoxifen inducible K14-Cre\textsuperscript{ERT} transgene to specifically label limbal and corneal epithelial stem/progenitor cells\textsuperscript{26}. Note that there may be differences between strains, as Di Girolamo and coworkers reported limbal specificity with no corneal expression for their K14-Cre\textsuperscript{ERT}\textsuperscript{27}. An advantage to using inducible transgenic Cre mice is that it allows the induction of lineage tracing at versatile time frames.

2. **Choose a Suitable Br Mouse Strain**

   1. Choose the appropriate Br cassette depending on the research question and available Cre line\textsuperscript{16,18,20}.
      1. If a non-inducible Cre mouse strain is used, choose a Br cassette that does not allow continuous DNA inversions/excisions and thus give rise to dead-end products (for example Br 1.0 or Br 1.1)\textsuperscript{16}.

3. **Cross the Transgenic Lines of Interest**

   1. For the protocol shown here, cross the homozygous R26R-Confetti strain (Br 2.1/Br 2.1) with the homozygous Cre\textsuperscript{ERT} strain (Cre\textsuperscript{ERT}/Cre\textsuperscript{ERT}) to obtain hemizygous offsprings (Br 2.1+/+; Cre+/+), that are ready for mono-allelic lineage tracing as they all contain a single Br 2.1 allele and a single Cre allele. Note: To increase the efficiency of labeling, one could generate mice that contain 2 Cre alleles (Br 2.1++; Cre+/Cre), while two Br 2.1 alleles (Br 2.1/Br 2.1; Cre+/+) would increase color combinations (as detailed in \textsuperscript{24}).
4. Cre-recombinase Induction

Note: The administration of Tamoxifen in these experiments was carried out by daily intraperitoneal injection, for the duration of 3-4 days, in order to induce the translocation of CreERT recombinase into the nucleus of limbal and corneal basal K14-positive stem/progenitor epithelial cells. The following protocol can be used for the injection of four mice. Alternatively, topical application of Tamoxifen to the ocular surface yields higher efficiency. The Tamoxifen solution can be kept in the dark at 4 °C for up to one week. Warm the solution before use.

1. Weigh 100 mg of Tamoxifen and dissolve it in 5 ml of corn oil to form a 20 mg/ml tamoxifen solution.
2. Inject 200 µl of Tamoxifen (4 mg) solution intraperitoneally to 8 weeks old mice for 3-4 consecutive days.

Note: The Tamoxifen solution can be kept in the dark at 4 °C for up to one week. Warm the solution before use.
3. For topical application: weigh 60 mg of Tamoxifen and dissolve it in 1 ml of corn oil to form a 60 mg/ml tamoxifen solution. Vortex and set the solution in a shaking water bath maintained at 65 °C until it becomes clear leave it in the bath until use.
   1. Carefully apply 100 µl of the Tamoxifen solution to each ocular surface of the mouse.

5. Tissue Preparation for Imaging

1. Sacrifice animals using isoflurane (2% vaporized in oxygen) anesthesia (confirm proper anesthetization by the lack of reflexes) followed by CO₂ inhalation at appropriate time points following Tamoxifen injection and collect the relevant tissue.
   1. For these experiments, enucleate eyes at the following time points: 1, 4, 8, 12, and 16 weeks after Tamoxifen injection. Achieve enucleation by using curved forceps. Press the eyelids causing the eyeball to pop out of the eye socket. Place the forceps behind the eyeball holding the optic nerve. Gently pull the eyeball and detach it.
   Note: At this stage, as described below, tissues can be prepared for wholemount analysis of labeled cells. Regular frozen tissue sections can also be prepared, fixed in PFA and imaged as described below (see section 6 and 22).
2. Place each eye in a 60 mm dish filled with PBS.
3. Under a stereo-microscope using forceps hold the eye near the optic nerve with the cornea facing down and use a scalpel in order to remove the optic nerve, muscle and connective tissue and make a small incision in the posterior part of the eye.
4. Using spring scissors carefully enlarge the cut letting the lens pop out and remove the iris using forceps.
5. Flatten the cornea by making 4-5 radial incisions starting from the center towards the periphery using a scalpel.
6. Use a scalpel to cut excess peripheral tissue beyond the limbus.
7. Fix corneas in PFA 4% for 10 min, then briefly wash them with PBS.
8. Incubate samples with DAPI for 10 min and briefly wash with PBS.
9. Mount corneas on glass slides with the epithelial side facing up. Place a drop of mounting media on top of the cornea and cover with a coverglass. Let slides dry O/N in RT. Keep slides at 4 °C.

6. Confocal Microscopy and Imaging Analysis

1. Use a spectral confocal system which enables separation of YFP/GFP/CFP emissions. Set fluorescence excitation and emission wavelengths depending on the fluorescent proteins of the Br2.1 cassette. Choose the most suitable excitation wavelength for each protein aiming at minimizing cross excitation. Choose narrow emission ranges in order to minimize the leak of signals from the different proteins.
   1. For the Br2.1 cassette, use the following set up of fluorescence excitation (ex) and emission (em) as a starting point: DAPI — ex 405 nm/em 420–480 nm, CFP — ex 458 nm/em 470–500 nm, GFP — ex 488 nm/em 507–528 nm, YFP — ex 514 nm/em 529–540 nm, and RFP — ex 561 nm/em 575–615 nm.
2. In order to visualize large tissue areas with high resolution, acquire tile imaging. For the results shown in Figure 2A-B, image the entire cornea by obtaining an array of 12 x 12 images, i.e., acquire 144 fields (512 x 512) using 4 fluorescent channels in each field. Altogether collect 576 images and then merge.
3. To explore the localization of tagged cells in different depths and structures of the tissue, acquire Z-stack images of different regions of interest. In order to image the full depth of the cornea, acquire 8 optical sections at 3 µm intervals. Orthogonal views of the sections collected as well as image projections based on maximum intensities can be constructed using conventional imaging softwares.

7. Corneal Injury Combined with Tamoxifen Induction

1. Anesthetize mice with isoflurane (2% vaporized in oxygen) and administer analgesics (10 mg/kg Carpofen, injected subcutaneously). Confirm proper anesthetization by unresponsiveness to toe-pinch.
2. Weigh 1 g of Tamoxifen powder and dissolve it in 5 ml of sterile DMSO to produce 200 mg/ml concentration solution.
3. Warm the Tamoxifen solution to 65 °C in a water bath until the solution becomes clear. Keep solution in the bath until use.
4. Apply eye ointment to the untreated contralateral eye to prevent dehydration during anesthesia. Do not leave an animal unattended until it has regained sufficient consciousness to maintain sternal recumbency. Do not return mice that had undergone eye wounding to the company of other mice until fully recovered. Mice should be closely monitored following the injury. In case they lose more than 10% of their body weight, suffer from corneal erosion, or discomfort, the experiment should be stopped. Repeat analgesics administration every 24 hr throughout the whole experiment.
5. Using a sterile syringe, without a needle attached to it; apply 200 µl Tamoxifen solution topically over the whole corneal surface of one eye of each mouse.

Note: The liquid solution solidifies quickly on the eye surface at RT. The effect of corneal injury depends on the severity of the wound. Following one application of Tamoxifen/DMSO solution no adverse effects are observed in the mice. In case of repeated applications corneal opacity can be observed.
6. Continue with tissue preparation and imaging (see sections 5 and 6) at relevant time points. For the results shown here, sacrifice the mice (as detailed in stage 5.1) one week post wounding (Figure 2 and 24).

**Representative Results**

Recently, we aimed at identifying the origin, survival and migration of stem cells and progenitor cells of the corneal epithelium. Accumulating evidence supports the dogma that the corneal epithelium is regenerated by stem cells located exclusively in the limbal niche, a ring shaped zone at the corneal periphery. This theory is supported by \textit{in vitro} and \textit{in vivo} data, and by clinical evidence that provided the basis for successful pioneering limbal stem cell therapy. However, this topic remained highly debated in the recent years due to a study based on limbal grafting experiments that suggested that the mouse limbus does not participate in corneal renewal. To test this interesting hypothesis, lineage tracing experiments using \textit{R26R-Confetti} mice were performed, to follow the fate of limbal and corneal epithelial stem/progenitor cells under steady-state conditions for up to 120 days. Eyes were enucleated and flat-mount corneas were analyzed by spectral confocal laser scanning fluorescence microscopy 10 and 120 days post Tamoxifen injection. As expected, small sporadic clusters of fluorescent cells were observed throughout the ocular surface 10 days after injection, while 1-4 months later (Figure 2B and 24), elongated streaks that extend from the limbus towards the corneal center slowly developed. This result suggests that the cornea is regenerated by cells that migrate from the limbus toward the center of the cornea. Next, corneal regeneration under wounding conditions was examined. In order to test the fate of K14-positive limbal/corneal cells under wounding conditions while inducing efficient cell tracking, we dissolved the Tamoxifen in dimethyl sulfoxide (DMSO), which causes corneal injury upon topical application. This way we managed to induce mild wounding if a single topical application was applied (Figure 2C), moderate wounding if additional DMSO alone treatment was applied in the day before Tamoxifen induction or severe wound when DMSO alone was topically applied in 2 sequential days prior to Tamoxifen induction.

In contrast to the thin limbal stripes that slowly developed and reached the corneal center within 4 months under steady-state conditions, cell migration following injury was rapid. Remarkably, long and thick limbal stripes were already developed under these conditions within 7 days. Interestingly, color diversity was sometimes minimal (Figure 2D). In our hands, occasionally, mainly red cells were identified in the whole cornea, indicating that color diversity can be biased towards specific fluorophores. This unwanted bias can be calibrated by dose response tests as reported previously in Br1.0L and in Br2.1.

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**Figure 1.** Schematic illustration of the \textit{R26R-Confetti} construct. (A) The R26R-confetti construct contains a CAG promoter for high expression levels, a loxP (black triangle)-flanked Neo\textsuperscript{R}-roadblock and the Brainbow2.1 cassette at the Rosa26 locus. The Brainbow2.1 cassette includes two head-to-tail loxP-flanked dimers. One dimer consists of nuclear-localized green fluorescent protein (GFP) and a reverse-oriented cytoplasmic yellow fluorescent protein (YFP, the inversed orientation is designated -PFY). The second dimer contains cytoplasmic RFP and a reverse-oriented membrane-tethered cyan fluorescent protein (CFP, the inversed orientation is designated -PFC). (B-E) Upon Cre-recombinase activation, different excision and inversion events may occur; possible outcomes are exemplified in B-E. The Cre activity induces stochastic rearrangement of the cassette leading to gene removals and/or inversions and thus results in the random expression of one of the four fluorescent proteins. Please click here to view a larger version of this figure.
Figure 2. Corneal lineage tracing under homeostasis and injury. R26R-confetti/K14-Cre mice were injected with 4 mg Tamoxifen as detailed in the protocol. In the indicated time points post induction flattened corneas were imaged (A-B). Corneal wounding coupled with Cre induction was achieved by topical application of Tamoxifen dissolved in DMSO (C-D). Mosaic images obtained by multichannel tiling spectral confocal microscopy are shown (A-D). In homeostasis, radial limbal stripes of slow migrating cells extended between the limbus and the corneal center. These stripes developed slowly, reaching the corneal center within 4-5 months (B). Clusters of corneal cells were evident as well (white arrows, B). In contrast, large limbal streaks appeared within a single week under wounding conditions (C). An example for bias of color diversity towards red cells (D). The limbal-corneal border is annotated by a dashed line. Scale bar is 500 µm. Abbreviations: CFP, cyan fluorescent protein; GFP, green fluorescent protein; RFP, red fluorescent protein; YFP, yellow fluorescent protein. This figure has been modified from 24. Please click here to view a larger version of this figure.

Discussion

Lineage tracing experiments have been conducted for many years. The recent technological improvements and the emergence of the Confetti mouse strains opened new avenues for research. Today, researchers can benefit from a large number of commercially available tissue-specific Cre lines, knockout mice and transgenic mice. This provides an opportunity for designing versatile experimental systems for addressing scientific questions with basic expertise, avoiding laborious practice, while providing robust and reliable data.

R26R-Confetti mice are an elegant tool for efficient tracking and studying the nature and behavior of cells in a living organism. The system is easy to establish and it allows non-invasive lineage tracing of single cells during embryogenesis, stem cell regeneration under homeostasis, or under different circumstances such as injury, inflammation and cancer. The obtainable data provides a robust description of the survival of targeted cells, clonal cell expansion and cell migration, in a quantifiable manner. A critical step would be to choose the appropriate genetic mouse strain that can reliably permit efficient tissue specific labeling at a precise developmental stage. One limitation of this system is that for monitoring a living organism, the tissue must be accessible and transparent. Similarly, tracking a thick tissue within a living animal may be only facilitated by two-photon or multi-photon microscopy. However, cell tracking is possible on postmortem tissue sections and perhaps the intact tissue may be studied after it has been transformed to become transparent by the CLARITY method32,33. Another limitation to be considered...
is that although adjacent cells that express the same fluorophore likely belong to the same clonal lineage, it is also possible that they may be derived from independent cell origin that randomly expressed the same fluorescent gene. This possibility becomes very unlikely when using multiple Br alleles to allow numerous color combinations.

In the future, combining the Confetti system with available genetic tools (i.e., knockout, knockin and transgenic mouse models) will permit the study of genes and their mutations in health and pathology. Likewise, lineage tracing under different system perturbations (i.e., stem cell niche destruction, laser mediated cell ablation, drug treatment) will bring new insights onto the involvement of signaling pathways in cell fate decisions. Ultimately, the combinatorial use of fluorescent and bioluminescence labeling, genetic reporters of signaling pathways and cutting edge microscopy will provide researchers a robust system to learn how single cells respond to their surrounding in their native environment.

Disclosures
The authors have nothing to disclose.

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