Genetic Techniques for Cell Lineage Tracing in the Nervous System

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Introduction

Deriving lineage relationships between cells in a developing organism, or between an early dividing cell of unknown potential and its descendants, has been a long-standing interest in developmental biology. In recent years, many new methods have emerged to enable cell lineage tracing with increasing resolution, leading to substantial biological insights. In model organisms, novel cellular labels, such as barcoded retroviral libraries (Gerrits et al., 2010) and a rainbow of available fluorescent proteins (Cai et al., 2013), have increased the number of founder cells that can be uniquely labeled and traced. Unlike most early cellular tracers, labels inserted into the genome can permanently mark lineages in a variety of experimental organisms without being diluted by cell division, and these modifications are facilitated by genome editing technologies (Hsu et al., 2014). In addition, recent advances in sequencing have enabled naturally occurring somatic mosaic mutations to be used as lineage marks in both cancerous tissue (Navin et al., 2011; Wang et al., 2014) and normal tissue (Behjati et al., 2014; Lodato et al., 2015), illuminating a future in which lineage tracing moves from experimental organisms into humans.

Prospective Methods of Lineage Tracing

A classic genetic approach to cell lineage analysis is performed by labeling a single founder cell and tracing its progeny over time. This prospective method has been used since biological dyes mapped the fate of cells within chicken and mouse embryos in early observational studies (Beddington, 1981; Serbedzija et al., 1989), and continues to be used in current lineage tracing experiments. Whereas early developmental studies hoped to achieve clonal labeling by microinjecting small amounts of dye into an area of interest, advancements in genetic tools for prospective lineage tracing now allow for far greater cell and tissue specificity, recombinase-based intersectional analyses, and single-cell resolution.

Sparse retroviral labeling for lineage tracing

Since the advent of recombinant DNA technology in the late 1980s, retroviral libraries containing reporter transgenes such as β-galactosidase (*β-gal*) and green fluorescent protein (*GFP*) have been used for cell labeling and lineage tracing in vertebrate animal models (Turner and Cepko, 1987; Frank and Sanes, 1991). Retroviral vector–mediated gene transfer allows viruses to introduce recombinant DNA into a host cell's genome. The integrated exogenous DNA is then inherited by all descendants of the infected cell. The DNA encodes a histochemical or fluorescent protein that can be easily assayed to label cells of a "clone" and elucidate cell fate choices within that clone. Histological and morphological analyses of the progeny of virally infected cells allow for *post hoc* fate mapping within a clonally related cell population.

Sparse retroviral infection has also been used in live cell imaging of progenitors and their progeny in organotypic slice culture. Mouse, ferret, chimpanzee, and human progenitors have all been analyzed using time-lapse imaging. Individual progenitors labeled by fluorescent reporter genes are visualized using confocal microscopy for multiple cellular divisions. At the end of the imaging experiment, immunohistochemistry and cellular morphology can then be used to analyze cell fate within the imaged clone (Noctor et al., 2001, 2004; Brown et al., 2011; Gertz et al., 2014; Dehay et al., 2015). Although *ex vivo* organotypic culturing conditions closely mimic the *in vivo* cellular environment, such experiments typically can be performed for only a few days at most, and so cannot typically relate clonal relationship to adult structure.

Sparse retroviral labeling requires that clonality be inferred based solely on proximity of cells expressing a reporter gene. Early studies in the cerebral cortex soon showed that sibling cells dispersed widely from one another in some clones (Walsh and Cepko, 1988). To analyze such widespread clones, the first retroviral libraries were developed, encoding the *lacZ* gene as a reporter, but also short DNA fragments to act as barcode tags (Walsh and Cepko, 1992). Clonal relationships were then directly revealed by PCR amplification of the integrated barcode tags from cells dissected from tissue sections, rather than being inferred based on proximity alone. Cells derived from a common progenitor share the same DNA tag at the vector integration site regardless of their patterns of migration, whereas clonally unrelated cells harbor different barcodes. The first library of a hundred tags soon expanded to a thousand tags (Walsh and Cepko, 1993; Reid et al., 1995) and then to essentially unlimited complexity using random oligonucleotide barcodes of identical size but distinct sequence (Golden et al., 1995; Fuentealba et al., 2015).

Advancements in transgenic animal lines have also extended the applications of retroviral genetic tagging and fate mapping. Cell-type specificity can now be achieved with transgenic mouse lines expressing virus receptors under the control of a cell-type-specific promoter (Harwell et al., 2015; Mayer et al., 2015). Only dividing cells that contain the virus receptor can

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be infected and express the reporter gene or barcode, allowing for more-precise viral targeting. Barcode tags can then be recovered using fluorescence-activated cell sorting (FACS) by the fluorescent reporter transgene, or laser capture microdissection (LCM) techniques that can preserve cellular position within the infected tissue for future reconstruction and analysis.

Although retroviral library labeling is useful for determining lineage relationships both *in vivo* and, it has some considerations and limitations: (1) only cells with the capacity to divide will propagate the barcode to progeny, (2) retroviral vectors typically spontaneously silence, so many retrovirally transfected cells are no longer histochemically labeled even though their DNA can be detected in the tissue, and (3) barcode tag recovery from single cells can prove challenging (Mayer et al., 2015). To circumvent the possibility of spontaneous retroviral silencing, new studies have been combining retroviral library labeling with RNA-sequencing (RNA-seq) technology. These studies not only recover barcodes to trace clonal lineage relationships but can also elucidate cell type using transcriptomics in sparse or heterogeneous cell populations (Lu et al., 2011). This valuable advance allows for the overlay of phenotypic cell identity with genetic lineage information for a more comprehensive view of clonal relationships.

Plasmid transfection labeling for lineage tracing

In addition to viral infection, reporter transgenes for cell labeling and fate mapping can be introduced into cells using DNA plasmid transfection. Lipofection, a common lipid-based system, has been used to transfect the developing *Xenopus* retina and to trace retinal cell fate *in vivo* (Holt et al., 1990). Electroporation, an alternative nonviral delivery method, has been used to deliver reporter transgenes encoding fluorescent proteins to trace cells both *in vitro* and in various vertebrate animal models (Fukuchi-Shimogori and Grove, 2001; Emerson and Cepko, 2011). Reporter gene plasmids can be injected into the developing brain's ventricles and introduced into neural progenitors lining the ventricular wall by electrical pulses. A reporter transgene, such as *GFP*, is then carried episomally by the progenitor cell and passed on to subsequent daughter cells. Unlike retroviral labeling, however, plasmid DNA is not integrated into the progenitor's genome and becomes diluted or inactivated in progeny after serial cellular divisions. Plasmid electroporation techniques, therefore, are transient and fail to label the entire lineage (LoTurco et al., 2009).

A solution to plasmid loss or inactivation is a DNA transposon system, which stably integrates the reporter transgene into the progenitor's genome. Transposon systems include Mos1, Tol2, Sleeping Beauty (SB), and piggyBac (PB), which all use a dual-plasmid approach with a "cut-and-paste" mechanism (Wu et al., 2006; VandenDriessche et al., 2009; Yoshida et al., 2010). The typical transposon system includes a donor plasmid containing the reporter transgene of interest and a helper plasmid that expresses the transposase. The donor plasmid includes terminal repeats flanking the transgene, which allows for genomic integration by the transposase. The transgene is then propagated to all progeny within the lineage, but the transposase (like any episomal plasmid) will be diluted over cellular divisions. Donor and helper plasmids can be driven by different promoters, allowing for cell-type specificity and genetic intersectional analyses. Compared with the other transposon systems, PB has a more precise cut-and-paste mechanism, higher transposition efficiency, and a larger cargo capacity (Chen and LoTurco, 2012). These attributes have made the PB transposon system particularly popular. In addition, PB transposase can be co-electroporated with multiple fluorescent reporter constructs, each driven by a cell-type-specific promoter. In this experimental design, multiple lineages can be examined in a single animal (Siddiqi et al., 2014). PiggyBac has been successfully used in multiple mammalian cell lines and in combination with *in utero* electroporation (IUE) to trace and manipulate cell lineages in animal models (Ding et al., 2005; Wilson et al., 2007; Woltjen et al., 2009; Siddiqi et al., 2014).

The piggyBac transposon plasmid system allows remarkable flexibility and cell-type specificity, but as with any random genomic insertion event, the precise location or number of transposition occurrences introduces a risk of confounded results due to mutagenesis. Transposition of the reporter transgene may cause endogenous genes at or near the insertion site to become unintentionally dysregulated. One study, however, found no evidence of mutagenesis by transposon insertion in cells labeled with the PB IUE method (Chen and LoTurco, 2012). Transposase plasmid systems are a remarkable tool for transgenesis and cell lineage tracing in both classically genetically modifiable animal models, such as mice, and otherwise non–genetically tractable animals, such as the ferret.

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Genetic recombination for lineage tracing

Cell lineage tracing by genetic recombination leverages the expression of recombinase enzymes in a cell-specific or tissue-specific manner to activate expression of a conditional reporter gene. Two genetically encoded, site-specific recombination systems include Cre-loxP and FLP-FRT. In the Cre-loxP system, mice are engineered to express Cre recombinase under the control of a chosen promoter, limiting Cre expression to a specific tissue or cell type (Orban et al., 1992). These mice are then crossed with a second mouse line in which a reporter transgene, such as *lacZ* or *GFP*, is preceded by a loxP-flanked transcriptional stop cassette. In cells expressing Cre recombinase, the STOP sequence is excised, and the reporter transgene is expressed. Temporal control of recombination can be gained by using an inducible Cre system, which selectively activates Cre under promoters that are also active at undesired time points such as embryogenesis. In an inducible system, Cre recombinase is fused to the human estrogen or progesterone receptor and activated only with the presence of an anti-estrogen such as tamoxifen or an anti-progestin, respectively. A pulse-chase strategy with an inducible Cre system can be used to determine lineage relationships. Leakiness, however, is a common problem of inducible Cre systems; nonetheless, these inducible systems have been used for lineage tracing in many adult tissues.

Dual or multicolor reporter lines have become increasingly popular for tracing cell lineage relationships. Mosaic analysis with double markers (MADM) uses a Cre-loxP system to express GFP and red fluorescent protein in cell populations of interest (Zong et al., 2005). Before recombination, no reporter transgene is expressed, but after Cre recombinase is activated, one or both transgenes are reconstituted. Green, red, or double-labeled yellow cells are generated depending on the recombination and the chromosomal segregation type. MADM can be used with cell-type-specific and inducible Cre systems to provide single-cell resolution and to more precisely examine progenitor division patterns (Zong et al., 2005; Hippenmeyer et al., 2010; Bonaguidi et al., 2011; Mayer et al., 2015). Multicolor lineage tracing is also possible with recent mouse reporter lines, including Brainbow and Confetti (Livet et al., 2007; Snippert et al., 2010). The Brainbow mouse lines harness stochastic Cre-mediated recombination using incompatible loxP sites to drive combinatorial expression of fluorescent reporter transgenes. Cells expressing a particular color share a common lineage. A modified line, the Confetti mouse, ubiquitously expresses Cre from the ROSA26 locus and has been used to trace individual stem cell lineages in the mouse intestinal crypt (Snippert et al., 2010). Owing to the expression of a multitude of unique colors, costaining with antibodies to determine protein expression within Brainbow or Confetti mice is nearly impossible. Endogenous fluorescence of the reporter genes, however, can be used for imaging clones. Advancements in microscopy, such as the two-photon microscope, continue to make these lines an attractive choice for *in vivo* cell lineage tracing.

Recent methodological advances in prospective lineage tracing

Innovations in both microfluidic platforms and genome editing strategies have also recently been used to prospectively trace cell lineage. Microfluidic technologies allow for capture and culture of single progenitor cells and up to five generations of their progeny on a single chip. *In vitro* time-lapse imaging for both division kinetics and identification of lineage relationships can be coupled with on-chip immunohistochemistry to assess cell fate within the captured clones. Clones can also be retrieved after culturing for single-cell transcriptomics with known lineage relationships. Kimmerling et al. (2015) used this microfluidic trap array technology, paired with single-cell RNA-seq, to look at both interclonal and intraclonal variability in activated $CD8^+$ T cells; they demonstrated that lineage-dependent transcriptional profiles corresponded to functional cellular phenotypes. This study was the first to link single-cell transcriptomics with cell lineage history (Kimmerling et al., 2015).

Recently, CRISPR/Cas9 genome editing technology (CRISPR signifies clustered regularly interspaced short palindromic repeats; Cas9 is a class of RNA-guided endonucleases) has been applied to trace and synthetically reconstruct cell lineage relationships in complex, multicellular organisms. McKenna et al. developed genome editing of synthetic target arrays for lineage tracing (GESTALT), a highly multiplexed method that uses barcodes composed of multiple CRISPR/Cas9 target sites (McKenna et al., 2016). These barcodes progressively and stably accumulate unique mutations over cellular divisions and can be recovered by targeted sequencing. Cell lineage relationships are determined based on the pattern of shared mutations among analyzed cells. While prospective in the sense that the barcode is introduced at the start of the experiment, the GESTALT method also parallels retrospective,

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somatic-mutation-based tracing, discussed below. The incrementally edited barcodes from thousands of cells were used in large-scale reconstructions of multiple cell lineages within cell culture and zebrafish. Although precise anatomical position and cell type of each assayed cell cannot be determined with this method, this published study and others in progress demonstrate the potential for cumulative and combinatorial barcode editing in prospective lineage tracing of whole organisms (Junker et al., 2016; Kalhor et al., 2016; McKenna et al., 2016). Advances during the past 30 years, since the advent of genetic barcoding and recombinase-based transgenic animals, have allowed prospective cell lineage tracing experiments to not only uncover clonal relationships at the single-cell level but also map cell fate choices in a wide variety of cells, tissues, and model organisms.

Retrospective Methods of Lineage Tracing

It has only recently become possible to harness naturally occurring mutations to retrospectively infer cell lineage information, owing to advances in genome sequencing. Like prospective lineage tracers in model organisms, somatic mutations indelibly mark the progeny of the dividing cell in which they occurred, and the cells bearing these naturally occurring lineage marks can be analyzed later to reconstruct the genealogy of organs and cell types (Salipante et al., 2010). To use naturally occurring somatic mutations for lineage tracing, it is first necessary to discover mutations shared among multiple cells of that individual; however, somatic mutations are difficult to identify by sequencing a mixed population of cells at conventional depths, as they are low-frequency by nature. Nonetheless, the declining cost of deep next-generation genome sequencing and the advent of single-cell genome sequencing have made it possible to discover rare mutations that mark minority lineages within a larger cellular population (Shapiro et al., 2013). These variants—from the least frequently somatically mutated to the most—include retrotransposons, copy number variants, single-nucleotide variants (SNVs), and microsatellites. The different rates at which these variants occur in somatic tissues allow lineage tracing experiments to be conducted at different levels of granularity according to the types of variants, tissue, and disease state selected. Single-cell genome sequencing promises to revolutionize lineage tracing in humans, although potential technical artifacts and complications must be considered when planning a single-cell genome sequencing experiment. Critically, whole-genome sequencing currently requires considerably more DNA than the 6 pg present in a single cell, necessitating a presequencing genome amplification step that may introduce errors (Grün and van Oudenaarden, 2015; Gawad et al., 2016).

Somatic mutations for lineage tracing in normal tissue

Endogenous retroelements, principally including long interspersed nuclear element–1 (LINE-1 or L1), compose much of the human genome; L1 elements alone make up nearly one-fifth of the genome (Ostertag and Kazazian, 2001). Some of these L1 elements retain the ability to mobilize in humans and can insert into a new genomic location during somatic cell division (Muotri et al., 2005). This mobilization has raised substantial interest in their potential contribution to somatic diversity, especially within complex tissues like the brain (Erwin et al., 2014). Estimates of L1 mobilization frequency derived by sorting single neurons, amplifying the whole genome, and analyzing L1 retrotransposition at a single-cell level (Evrony et al., 2012) suggest there are fewer than one somatic insertion per neuronal genome on average (Evrony et al., 2012). A single-neuron whole-genome sequencing study confirmed the low rate of L1 retrotransposition events but also illustrated the striking spatial distribution patterns of clonal retrotransposition events, providing strong proof of principle for the use of spontaneous somatic L1 events for lineage tracing (Evrony et al., 2015).

SNVs are a significant source of evolutionary and disease-causing mutations, yet they can also occur very frequently in noncoding portions of the genome without having functional effects on somatic cells. Somatic SNVs represent a rich source of lineage-marking mutations because they are both abundant and frequently functionally neutral. Indeed, work in mouse stomach, intestine, and prostate (Behjati et al., 2014), mouse brain (Hazen et al., 2016), and human brain (Lodato et al., 2015) suggests that somatic SNVs can be identified from single cells or clones and used to reconstruct developmental lineages; in one study, 9 of 16 sequenced neurons, and 136 of 226 total neurons from the same area of cortex, could be placed in a lineage tree with four independent clades that diverged before gastrulation. One clade contained a nested set of 11 somatic mutations, which were progressively regionally restricted across the brain and present in progressively decreasing frequency in bulk tissue (Lodato et al., 2015). These results suggest that analysis of such nested mutations might enable the analysis of the progressively narrower lineage trees characterizing the developing embryo.

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The most frequently mutated somatic loci are microsatellites (Ellegren, 2004). Because of the instability of microsatellite repeats, analysis of all microsatellite locations in the genome is predicted to be capable of reconstructing the entire cell lineage tree of an organism (Frumkin et al., 2005), using methods adapted from organism-level phylogenetic analysis (Salipante et al., 2010). Like microsatellites, the polyadenylated tracts following somatic L1 retrotransposition events are subject to frequent polymerase slippage, and therefore, lineages defined by a somatic L1 retrotransposition event can be further delineated by analyzing poly-A tail polymorphisms (Evrony et al., 2015).

Perspective

When designing a lineage tracing experiment, it is important to consider the strengths and weaknesses of prospective and retrospective approaches. For prospective lineage tracing, there must be genetic access to the population in question, whether by a regionally directed method such as viral injection or electroporation, or by population-specific marker lines or promoters. Because prospective lineage tracing depends on labeling and follow-up analysis, its use is restricted to experimental organisms and cell-culture systems. Alternately, retrospective lineage tracing can investigate lineage directly in human tissue, allowing unprecedented access to lineage information relevant to human development and disease. Currently, retrospective lineage tracing relies heavily on sequencing, frequently of single cells, and is therefore lower-throughput and more expensive than most prospective methods. Although emerging prospective lineage systems are engineering revolutionary ways to investigate lineage in model organisms, it will always be necessary to retrospectively map lineage in naturally occurring tissues without engineered lineage marks.

No longer limited to tracing a small number of cells with serially diluted dyes, biologists can now access a variety of methods for tracing lineage forward from the application of a genetic label. Additionally, recent advances in sequencing—particularly genome sequencing of single cells—allow lineage tracing to be performed retrospectively, reconstructing lineage decisions that occurred well before sequencing. A hundred years after the first investigations of cell lineage, developmental biologists have built a tremendously enriched genetics toolkit for examining the developmental fate decisions that construct a whole organism.

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