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BRAIN RESEARCH

Research Report

# Statistical analysis of data from retroviral clonal experiments in the developing retina

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ABSTRACT

Retroviral lineage studies have been widely used over the past decade to study retinal development in vivo and in explant culture [Donovan S.L., Dyer, M.A., 2006. Preparation and Square Wave Electroporation of Retinal Explant Cultures, Nature Protocols 1, 2710–2718; Donovan, S.L., Schweers, B., Martins, R., Johnson D., Dyer, M.A., 2001. Compensation by tumor suppressor genes during retinal development in mice and humans, BMC Biol 4 , 14; Dyer M.A., Cepko, C.L., 2001. p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations, J. of Neurosci 21, 4259–4271; Dyer M.A., Cepko, C.L., 2000. p57 (Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina, Development 127, 3593–3605; Dyer, M.A., Livesey, F.J., Cepko C.L., Oliver, G., 2003. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina, Nat Genet 34, 53–58]. These approaches can provide important data on the proliferation, cell fate specification, differentiation and survival of individual neurons and glia derived from single infected retinal progenitor cells. In some experiments, these parameters are compared in retinae from animals with different targeted deletions or transgenes. Alternatively, the effect of ectopic expression of virally encoded transgenes may be studied at the level of individual retinal progenitor cells in vivo and in explant culture. One of the challenges with interpreting retroviral lineage studies is determining the statistical significance of differences in the proliferation, cell fate specification, differentiation of survival of retinal progenitor cells between experimental and control samples. In this study, we provide a clear step-by-step guide to the application of statistical methods to retroviral lineage analyses actual data sets. We anticipate that this will serve as a guide for future statistical analyses of retroviral lineage studies and will help to provide a uniform standard in the field.



## 1. Introduction

In vivo retroviral lineage studies in the developing retina were first described by Turner and Cepko (1987). They developed a replication-incompetent retroviral shuttle vector that could be used to produce retroviral stocks of sufficient titer for in vivo retinal infection using newborn rat pups (Turner and Cepko, 1987). In the initial study, a β-galactosidase reporter gene was used and subsequent versions of these viral vectors incorporated human placental alkaline phosphatase and nuclear

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β-galactosidase reporter genes (Dyer and Cepko, 2000; Dyer and Cepko, 2001b; Fields-Berry et al., 1992) (Figs. 1A, B). Alkaline phosphatase is ideally suited for visualizing the morphology of infected retinal neurons and glia because it labels their membrane (Figs. 1C, D) and nuclear β-galactosidase is ideally suited for studies focused on retinal progenitor cell proliferation because the number of nuclei in clones of cells derived from infected retinal progenitor cells can be readily identified (Figs. 1E, F). These and similar techniques have been used over the past decade to study retinal development in vivo and in explant culture (Donovan and Dyer, 2006; Donovan et al., 2006).

The advantage of performing lineage studies in the developing retina is that the daughter cells from individual infected retinal progenitor cells do not migrate laterally so clonal boundaries can be unambiguously identified (Fields-Berry et al., 1992). More recent versions of these replication-incompetent retroviral vectors have incorporated an IRES-reporter gene configuration which allows investigators to ectopically express different genes along with the reporter gene (Fig. 1C) (Cepko et al., 1998; Furukawa et al., 1997). In this way, researchers can study the effect of ectopic gene expression on retinal progenitor cell proliferation, cell fate specification, differentiation or survival in vivo using clonal analysis. Versions of these viral vectors that express Cre recombinase have also been used to conditionally inactivate floxed genes in single infected retinal progenitor cells and similarly analyze the effects on proliferation and development of the daughter cells (Zhang et al., 2004). Some investigators have used retroviruses to study the development of the retina in mice carrying targeted deletions of different genes (Dyer and Cepko, 2001a; Dyer et al., 2003). By combining retroviral-mediated ectopic expression with mouse strains carrying floxed or knockout alleles of genes believed to be important for retinal development, this system provides a great deal of flexibility in studying the genetic basis of retinal development.

In this article, we will discuss the statistical consideration for experiments that fall into two different classes. The first type of experiment utilizes different retroviral vectors in the left and right eyes of mice with the same genetic background. For example, one eye of a mouse carrying a floxed allele of a

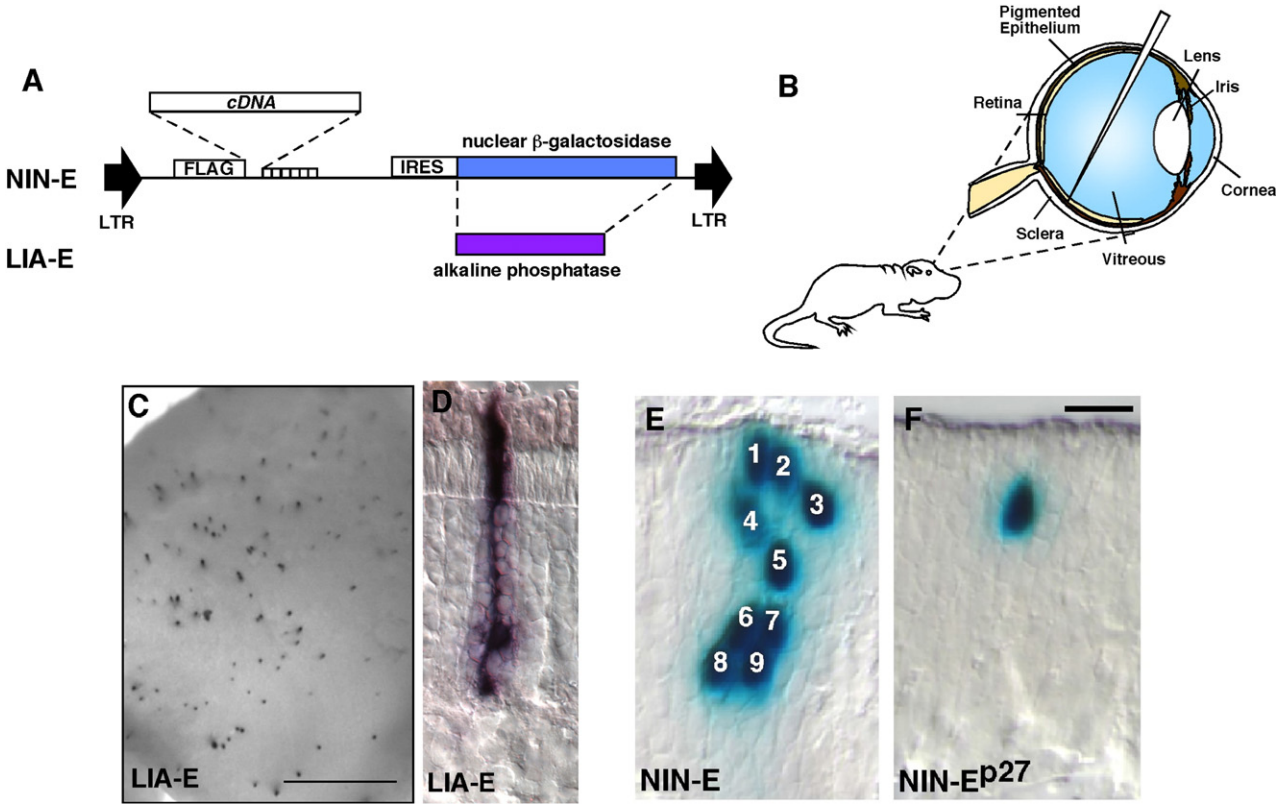


**Fig. 1 – Retroviral lineage analysis in vivo and in explant culture. (A) A series of replication incompetent murine retroviral vectors were developed that are suited for expressing a reporter gene such as alkaline phosphatase or nuclear β-galactosidase along with a cDNA of interest. These vectors also encode a FLAG epitope tag and a 6×His tag for protein analysis. (B) For in vivo retroviral infection of the developing retina, P0 mouse pups receive a subretinal injection of 0.5 μl of viral stock with a titer of approximately $0.5–1.0 \times 10^7$ infectious particles per ml. 3 weeks later after retinal development is complete, the retinae are isolated and stained for alkaline phosphatase expression as whole tissue samples (C). Cryosectioning of these stained retinae reveals the neuronal morphology of individually infected retinal cells that were derived from single infected retinal progenitor cells. (D) A rod photoreceptor labeled with alkaline phosphatase is shown. (E, F) For analyzing retinal progenitor cell proliferation in retinal explant cultures, we prefer the NIN-E retrovirus that encodes nuclear β-galactosidase. Even large clones can be readily scored for the size of the clones. (F) An example is shown of a virus that ectopically expresses p27 which induces premature cell cycle exit and as a result smaller clones. Abbreviations: IRES, internal ribosome entry site. Scale bars: 1 mm in panel C and 10 μm in panel F.**