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Use of Lentiviral Vectors to Deliver and Express Bicistronic Transgenes in Developing Chicken Embryos

Susan L Semple-Rowland and Jonathan Berry

Department of Neuroscience, University of Florida McKnight Brain Institute, Gainesville, FL 32610 0244, United States

Abstract

The abilities of lentiviral vectors to carry large transgenes (~ 8Kb) and to efficiently infect and integrate these genes into the genomes of both dividing and non-dividing cells make them ideal candidates for transport of genetic material into cells and tissues. Given the properties of these vectors, it is somewhat surprising that they have seen only limited use in studies of developing tissues and in particular of the developing nervous system. Over the past several years, we have taken advantage of the large capacity of these vectors to explore the expression characteristics of several dual promoter and 2A peptide bicistronic transgenes in developing chick neural retina, with the goal of identifying transgene designs that reliably express multiple proteins in infected cells. Here we summarize the activities of several of these transgenes in neural retina and provide detailed methodologies for packaging lentivirus and delivering the virus into the developing neural tubes of chicken embryos *in ovo*, procedures that have been optimized over the course of several years of use in our laboratory. Conditions to hatch injected embryos are also discussed. The chicken-specific techniques will be of highest interest to investigators using avian embryos, development and packaging of lentiviral vectors that reliably express multiple proteins in infected cells should be of interest to all investigators whose experiments demand manipulation and expression of multiple proteins in developing cells and tissues.

Keywords

development; chicken; retina; nervous system; 2A peptide; dual-promoter

1. Introduction

Understanding the molecular mechanisms that control the growth, differentiation, and regeneration of developing cells or tissues often requires manipulation of the expression of specific genes. Several different techniques have been developed to alter the expression of

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Corresponding Author: Susan L Semple-Rowland, PhD Department of Neuroscience University of Florida McKnight Brain Institute Box 100244 Gainesville, FL 32610-0244 rowland@ufl.edu Phone: 352-392-3598 Fax: 352-392-8347.
Author email addresses: Semple-Rowland – rowland@ufl.edu Berry - jberry.09@ufl.edu

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either nucleic acids or proteins in cells, most of which involve delivery of specifically engineered transgenes. Electroporation and viral vectors are two of the most commonly used techniques to deliver transgenes to developing tissues, each of which has unique advantages and disadvantages. When selecting a method to deliver transgenes to specific tissues and cell populations, it is important to consider the size of the transgene that is to be delivered, whether the targeted cells are dividing or quiescent, whether stable or transient DNA expression is required, and whether it is desirable to express multiple proteins in the targeted cells.

Electroporation uses an electric field to create temporary pores in the membranes of cells through which DNA is driven by the applied current. This approach, which was first used to introduce transgenes into chick embryos *in ovo* [1], continues to be a popular experimental method to alter gene expression in cultured cells [2], brain slices [3], and in intact developing embryos [4] because it is efficient, relatively inexpensive, and can be used to target expression of several genes to a variety of brain regions and neural cell types. Specific populations of cells can be targeted by electroporating embryos at specific developmental stages and by carefully selecting the orientation of the applied electrical field relative to the brain [5–7]. Specific neural cell types within these populations can be targeted by selecting promoters that exhibit cell-specific activity [8–10]. In addition, we and others have observed that the vast majority (>90%) of the cells within a tissue that is electroporated with a mixture of plasmids will contain all of the plasmids in the mixture, as evidenced by cellular expression of the transgenes carried by the plasmids [11]. Recent advances in techniques designed to control gene expression have opened the door to the development of several new, innovative cDNA constructs that when electroporated into cells either alone or in combination with complementary constructs, enable investigators to address research questions that cannot be easily addressed using other methodologies. Use of conditional vector systems that allow investigators to either permanently or reversibly turn on or off the expression of electroporated transgenes have proven to be particularly useful in studies of development [11]. A significant drawback to electroporation is that the transgenes delivered to the electroporated cells remain episomal and as such, can be significantly diluted or lost in actively dividing cell populations. Promising solutions to this problem in which transposons and transposase are used to induce integration of the electroporated transgene into the host cell's genome have been developed [12,13], solutions that should greatly increase the usefulness of electroporation.

Like electroporation, viral vectors have proven to be an extremely efficient method to deliver transgenes to cells. Retroviruses in particular have been widely embraced in studies of developing tissues because, unlike the transgenes carried by electroporated plasmid DNA that largely remain episomal, the transgenes carried by retroviruses integrate into the host cell's genome thereby ensuring that the transgenes are transferred to daughter cells. Several classes of retroviruses have been used in developmental studies, including a series of replication-competent and replication-defective vectors developed from the avian Rous sarcoma virus (RSV) [14–16]. These vectors have been most useful in studies of avian embryo development [17–20] but can also be used to infect proliferating mammalian cells if the specific receptor for the viral envelope protein of the virus is ectopically expressed in the

target cells [21–24] or if envelope genes of viruses that can infect mammalian cells are used when packaging these vectors [25,26]. It should be noted that mammalian cells infected with RSV-derived vectors cannot themselves produce virus. Transduction with RSV-derived vectors is limited to actively dividing cells, and the relatively small transgene capacity of these vectors (~ 4 Kb including promoter and poly A elements) precludes use of these vectors to deliver large genes or transgenes encoding multiple proteins. An additional caveat is that infection of chicken embryos with a particular RSV subtype (A–E), which is defined by the envelope glycoprotein encoded by the *env* gene, is dependent on whether the embryo has been previously exposed to virus expressing encoding the envelope protein. If previously infected, expression of the envelope protein by the cells blocks the ability of the RSV virus to bind to the cell surface receptors and infection of the cells. To avoid this problem, we have been able to obtain virus-free, fertile chicken eggs from Charles River that can be infected by all RSV subgroups. Even with these limitations, RSV-carried transgenes have been targeted to specific tissues by including tissue-specific promoters in the vector constructs [27,28].

In addition to the RSV-derived vectors, lentiviruses have been used to deliver transgenes to targeted tissues and cells. Lentiviruses, like RSV, are members of the *Retroviridae* family, but unlike RSV vectors, they can efficiently infect and integrate into the genomes of both dividing and non-dividing cells and they have a large transgene capacity (~8Kb), both of which make them particularly useful for applications requiring long-term sustained expression of large or multicistronic transgenes in target cells. Examples of use of these vectors include neural development studies to examine cell lineage in developing chicken retina [29], molecular mechanisms underlying song development in the finch [30], and migration of neuroblasts to the olfactory bulb in postnatal mouse brain [31]. We have taken advantage of the large capacity of these vectors to explore the expression characteristics of several dual promoter constructs [9,10] and bicistronic transgenes containing the porcine teschovirus-1 (PTV1), 2A cleavage sequence [32] with an eye toward obtaining reliable expression of multiple proteins in infected cells. Given the properties of lentivectors, it is somewhat surprising that these vectors have not taken center stage in studies of developing neural tissues. Our efforts to develop lentiviral vectors that reliably express multiple proteins should be of interest to investigators who want to manipulate the expression of multiple proteins in developing tissues. Here we provide a detailed description of our viral vector, our method to deliver lentivirus to the neural tubes of chicken embryos *in ovo*, and hatching injected embryos. In addition, we discuss the expression characteristics of multicistronic transgenes that we have constructed using ubiquitous and cell-specific promoters in the context of both dual-promoter and 2A cleavage peptide lentivectors in chicken retina.

2. Construction and packaging of lentivectors

2.1 Lentiviral vectors

2.1.1 Lentiviral vector backbone—The current pFIN lentivector backbone used in our studies was derived from the pTY lentivector [33] by inserting a central polypurine tract (cPPT)-DNA FLAP element upstream of the multiple cloning site [34] and a 2x250 bp chicken β -globin HS4 core sequence into the 3'-LTR [9]. The FLAP sequence has been

reported to improve the transduction efficiency of recombinant lentiviral vectors [35–37] and insertion of the insulator sequence into the 3' LTR produces an integrated transgene that is flanked by insulator sequences, sequences that have been shown to minimize transcriptional interference from genomic DNA flanking the integrated transgene [38].

2.1.2 Multicistronic lentiviral vectors—The relatively large transgene capacity of lentiviral vectors has made them attractive vehicles for delivery of complex, multicistronic transgenes to cells. In addition, they have proven to be particularly useful in studies of developing tissues because they are able to transduce both dividing and non-dividing cells and the transgenes they carry are integrated into the host cell's genome, a particularly useful feature if the infected cell is dividing and transfer of the transgene to daughter cells is desired. In recent years, we have constructed and examined the expression characteristics of several different multicistronic lentiviral transgenes with the goal of identifying vectors that reliably express the proteins they encode. Our efforts have been driven in part by our observation that only a small percentage (<10%) of neural cells exposed to mixtures of two lentiviruses are actually infected by both of the viruses in the mixture [10] and our goal to develop multi-protein therapies for inherited photoreceptor disease.

2.1.2.1 Use of cell-specific promoters: The ability to restrict expression of viral transgenes to specific cell types is a powerful tool. In many of our experiments we are interested in targeting expression of transgenes to specific cells in the neural retinas of chickens. Over the course of our studies we have characterized the activities of several different promoter fragments in developing and mature chicken retina, many of which were derived from the promoters of photoreceptor-specific genes (Table 1). One of our most important findings arising from these studies is that the cellular specificity of many promoters is well conserved across species. Since chicken is the primary animal model used in our laboratory, this observation served to dramatically increase the number of promoters that we test for use in our studies.

2.1.2.2 Dual promoter configurations: The transgene configuration that is most often used to express multiple proteins from a single transcript involves insertion of an internal ribosomal entry site (IRES) between the protein coding sequences. While this design can produce multiple proteins, we and others have found that the gene located downstream of the IRES is often either not expressed or is expressed at levels too low to be effective for many applications [39,40]. A possible solution to this problem, first described by Yu et al [40], is to replace the IRES with a second independent promoter, a configuration that these authors found yielded high, sustained expression of two genes in transduced stem cells.

We have built and characterized the expression properties of several dual promoter vectors in chicken retina [9,10]. The results of our experiments show that it is possible to construct dual promoter vectors that reliably express two proteins in a cell-specific manner; however, we also found that it is not always possible to predict the expression characteristics of these transgenes in infected cells based solely on the unique expression characteristics of the promoters used to construct them. One of the biggest problems we and others have encountered when designing transgenes carrying multiple promoters arises from promoter suppression, a phenomenon wherein the expression characteristics of one promoter are

altered by the presence of a second promoter [41,42]. In the most severe cases of promoter suppression, we found that the expression of one of the promoters in our dual-promoter transgenes was completely overridden by that of the second promoter. Interestingly, we found that it was possible, in some cases, to ameliorate this problem by reversing the order of the promoters in the transgene [9,10].

We have built and characterized the expression of several different dual promoter transgenes in chicken neural retina. In constructing these transgenes we have either paired two different cell-specific promoters, a cell-specific and ubiquitously expressed promoter, or two identical promoters (Table 2). We found that the expression characteristics of the individual promoters used in transgenes carrying two different cell-specific promoters were often preserved, although the levels of expression obtained from each could vary depending on the position of the promoter in the transgene. Pairing a cell-specific promoter with a ubiquitously expressed promoter produced mixed results. In some constructs, we found that the specificity of the cell-specific promoter was retained and in others, the activity of the ubiquitously expressed promoter superseded that of the cell-specific promoter. Reversing the order of these promoters in the transgene could, in some cases, re-instate the expression characteristics of the cell-specific promoters. Finally, we did construct several transgenes using two identical promoters, a design that is often not recommended because of the possibility of loss of transgene sequence through homologous recombination. We found that although these vectors could be packaged into virus, the levels of expression of the proteins encoded by the upstream cistrons were oftentimes lower than those obtained from the downstream cistron [32].

2.1.2.3 Use of 2A cleavage peptides: An alternative approach that we have successfully used to construct bicistronic transgenes expressing two proteins involves in-frame insertion of the PTV1 2A peptide cleavage sequence between the open reading frames encoding the proteins [32] (Table 3). Viral 2A cleavage sequences are short peptide sequences that are found in a range of mammalian and insect viruses that, when fused in frame between two cistrons, trigger co-translational 'ribosomal skipping', a process that produces equimolar quantities of the proteins encoded by the transcript [43,44]. Since their initial discovery, the small size and efficiency of "cleavage" of these sequences has continued to be exploited by several investigators interested in expressing multiple proteins in infected cells [44–46]. Readers are directed toward a recently published protocol series for additional information on the design, construction and verification of the cleavage of gene constructs containing 2A peptides [47–49]. Caveats to use of these peptides include the possibility that the fusion protein encoded by the transcript will not always cleave properly during translation, a process that can be affected by the nucleotide sequences immediately flanking the peptide. In addition, post-translational processing and intracellular processing of the encoded proteins can be altered as a result of the order that the cistrons appear in the transgene [50]. To try to improve cleavage of the 2A containing peptides translated from some of our transgenes, we introduced a furin cleavage site (RAKR) just upstream of the GSG linker-2A sequence (Table 3), an approach that has been successfully used by other investigators [51,52]. Addition of this sequence to our 2A vectors did not, in general, improve their performance and in fact seemed to interfere with cleavage. In general, our experience with

use of 2A peptides indicates that the performance of each construct must be thoroughly verified before use.

2.2 Lentiviral packaging

The HIV-1-based lentiviral vector system and packaging method that we use generates self-inactivating virus pseudotyped with the vesicular stomatitis virus G (VSV-G) glycoprotein [9].

2.2.1 Materials and reagents

Equipment

- Low speed centrifuge equipped with swinging buckets capable of spinning Centricon Plus-70 filter units
- Ultracentrifuge fitted with swinging bucket rotor
- Class II A/B3 Biological Safety Hood
- Cell culture incubator
- Optional: inverted fluorescent microscope

Plasmids

- pNHP vector - helper construct encodes Gag-Pol, Tat, Rev, Vpr, and Vpu and carries deletions of 5', U3, 5'UTR, part of env, nef, and the 3' LTR [53]. Vector available from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat# 4692) and from Addgene (plasmid 22500).
- pHEF-VSVG - encodes the vesicular stomatitis virus G (VSV-G) glycoprotein for pseudo-typing driven by human elongation factor alpha promoter [53]. Vector available from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat# 4693) and from Addgene (plasmid 22501).
- pFIN transducing vector carrying the transgene encoding protein(s) of interest. Several of the vectors described here can now be purchased for a minimal fee from Addgene (search term: Semple-Rowland). Vectors not banked at Addgene are available by request from the corresponding author. In addition, the sequences and maps for these vectors can be obtained by searching everyVECTOR© (<http://www.everyvector.com>) using the vector name.

Reagents and Supplies

- Pen/Strep (100X) (Invitrogen, Cat 15140-122)
- Geneticin (Gibco; Cat 10131)
- Fetal Calf Serum (FCS) – heat inactivated (Hyclone; Cat SH3007003HI)
- Trypsin, EDTA, 1X (Mediatech, Inc; Cat 25-053-CI)
- Superfect reagent (Qiagen,; Cat 301307)

- DMEM (high glucose + glutamine + sodium pyruvate) (Gibco; Cat 11995073)
- Gelatin (Fisher Scientific; Cat G7-500)
- Qiagen EndoFree Maxi kit (Qiagen; Cat 12362)
- 0.45 μ M Stericup-HV filter units (Millipore; Cat SCHVU01RE)
- Centricon Plus-70 Centrifugal filter units (Millipore; Cat UFC710008)
- 30 ml conical polyallomer ultracentrifuge tubes (Beckman; Cat 358126)
- Lenti-X qRT-PCR kit (Clontech; Cat 631235)

Solutions

- 293FT culture media – 10% FCS and 1X Pen/Strep in DMEM
- 1% gelatin w/v in sterile water

2.2.2 Virus Packaging procedure—This detailed protocol is currently used in our laboratory to package all of our lentiviral vectors. An abbreviated summary of this method has been previously published [9]. The procedure takes 4 days to complete from transient transfection of the 293FT cells to generating aliquots of the final virus for storage at -70°C until use. In most cases, the viruses that we prepare for use in our experiments are prepared using three T225 flasks that yield approximately 60–72 μ l of virus, which we store in 6 μ l aliquots at -70°C . The titers of our viruses are estimated using a Lenti-X qRT-PCR kit and typically average 2×10^{12} viral genomes per ml.

It is appropriate to briefly discuss safety issues that should be considered when working with lentivirus. The 3-plasmid system we use to package virus includes several features that greatly reduce the possibility that the virus produced is not replication competent. The most significant of these features is the deletion of essential genes that are required for replication from the packaging system. All of our experiments involving packaging and use of lentivirus are carried out using Biosafety Level II practices. While the later generation vector systems that have been developed incorporate several features that dramatically reduce the potential to generate replication competent virus, it is very important that researchers contact their environmental health and safety officers to discuss their planned experiments using this viral vector before initiating them. Links to current NIH policies concerning research involving recombinant DNA molecules and lentiviral vectors can be found at http://oba.od.nih.gov/rdna/nih_guidelines_oba.html.

1. Coat the bottoms of T225 flasks with thin layer of sterile 1% gelatin in a sterile safety hood. To do this, pipet 10 ml of gelatin solution into the first flask, tilt the flask to ensure coverage of the entire surface, remove the excess gelatin solution, and stand the coated flask up on end while coating the next flask. Continue this procedure until all flasks are treated. When all of the T225 flasks have been coated, return to the first flask and aspirate out the last drops of gelatin solution that have flowed off the coated surface. Allow flasks to air dry standing on end with caps removed in the safety hood. It is important to be sure that the gelatin is completely

dry to prevent it from detaching during use of the flask. We often coat the flasks on a Friday and allow them to dry in the hood until use on Monday.

2. Seed 293FT cells (Invitrogen, Carlsbad, CA) onto the gelatin-coated surfaces of the T225 flasks at a density of 3×10^7 cells per flask in a final volume of 26 ml media. As a general rule, we do not use 293FT cells that have been passaged more than 15 times. We have found that one T75 flask containing confluent 293FT cells yields a sufficient number of cells to seed one T225 flask at this cell density. The 293FT cells used for packaging should be grown in the absence of Geneticin. To collect the 293FT cells from the T75 flasks, remove the media from the flask, gently rinse cells using sterile PBS (without Ca^{2+} or Mg^{2+}), and loosen cells from flask surface by incubating cells in 1 ml trypsin at 37°C for 5 min. Add 6 ml media containing serum to the flask to deactivate the trypsin and suspend the cells in solution by triturating (~10 strokes using 10 ml pipet). Pool cells obtained from all of the T75 flasks into one flask and determine cell number/ml using a hemacytometer.
3. Incubate T225 flasks overnight at 37°C and 5% CO_2 until cells reach 80–90% confluency, which is usually achieved within 24 hours of seeding. If cell confluency is too low, transfection will be less efficient and the virus yield will be reduced.
4. Prepare DNA transfection mixture. All plasmid DNA used for viral packaging should be endotoxin-free. For each T225 flask, add 21.3 μg pNHP, 8.4 μg pHEF-VSVG, and 9.2×10^{11} copies of the transducing vector carrying your transgene to 1 ml of DMEM in a polystyrene tube. Briefly mix DNA solution and then add 150 μl Superfect. Mix gently and incubate for 10 min at room temperature without shaking. Following the incubation, add 12 ml culture media to each tube and mix thoroughly by gently pipetting the solution.
5. Aspirate media from T225 flask and replace with 12 ml of transfection mixture. Be sure that the volume of mixture covers the cells. If necessary, the volume can be increased slightly but optimal transfection occurs when the volume is kept to a minimum. Incubate the flasks with the transfection mixture at 37°C and 5% CO_2 for 4–5 hours.
6. At the end of the incubation period, remove the transfection mixture and replace it with fresh culture media (18 ml).
7. The virus containing media is collected two times –24 h and 40 h post-transfection. The media collected at each time point is centrifuged at $783 \times g$ for 5 min at 4°C in 50 ml polypropylene capped tubes to remove cell debris. The centrifuged media collected 24 h post-transfection is then transferred to clean 50 ml tubes and stored at 4°C until the next day. The quality of the transfection can be determined by examining the flasks using a fluorescent microscope if a fluorescent protein is encoded by the transgene and the promoter driving expression is active in 293FT cells. Another indicator of a successful transfection is the fusion of the 293FT cells resulting in the formation of multi-nucleated cell syncytia that is induced by expression of VSVG [54,55].

8. Once the 40 h post-transfection media is collected and centrifuged, pool the media samples collected at 24 h and 40 h and filter them through a 0.45 μ M Stericup-HV low protein-binding filter device.
9. Concentrate all but 26 ml of the filtrate using a Centricon-70 ultrafiltration column. Centrifuge the column assembly at $2500 \times g$ for 1 h at 4°C and recover the column retentate containing the virus according to the manufacturer's instructions. During centrifugation step, store the 26 ml of filtrate at 4°C until step 10.
10. Add the viral retentate collected from the Centricon column to the 26 ml of unconcentrated virus media (Step 9) by gently swirling the mixture, pipet the mixture into a 30 ml conical polyallomer tube, and centrifuge at $103,680 \times g$ in a SW28 rotor (Beckman) for 1.5–2.0 h at 4°C.
11. Carefully aspirate the media overlying the viral pellet. The viral pellets, which can often be seen at the bottom of the conical tubes, usually adhere to the bottom of the tube. To remove final few mls of media from the tube, tilt the tube 45 degrees and continue to aspirate media as it flows down the side of the tube away from the pellet.
12. Gently overlay the viral pellet in the conical tube with 25 μ l sterile DMEM (if virus prep was generated from 3 T225 flasks), seal the conical tube using Parafilm™, and store the virus at 4 °C overnight. We have found that this method of resuspending the viral pellet in solution minimizes loss of and damage to the viral particles that can occur if resuspension is carried out by pipetting. The next day, gently shake the viral solution on ice for 2 h using a rotating shaker, divide the resuspended virus into working aliquots, and store at –70 °C until use. If the titer of the virus is desired, an aliquot of the virus can be separated out for this purpose and either assayed or frozen until analyzed. Lentivirus should not be thawed and refrozen.

3. Chicken embryo injection and hatching

The following procedure has been developed by several members of our laboratory over several years with the goal of obtaining the highest hatch rates of injected embryos.

3.1 Materials

Equipment

- Glass micropipette puller
- 3-way micromanipulator
- Nanoject II (Drummond, Cat 3-000-204)
- Dissecting microscope
- Flexible arm microscope lighting system
- X-Acto extra fine razor saw blades (Elmer's Products, Inc., Cat X239)
- Foam egg holders lined with gauze pads

- Rocking egg incubator (Sportsman Incubator; GQF Manufacturing)

Reagents

- Light mineral oil

Solutions

- 0.03% Fast Green dye (w/v) in sterile DMEM

3.2 Incubation of pre-injection eggs

Incubate fertile, day 0 eggs vertically with the large end of the egg uppermost at 37.5 °C and 55–60% humidity without rotation until the embryos reach the desired stage of development for injection. In most of our experiments, our goal is to infect as many retinal progenitor cells as possible. We have found that good results can be obtained by injecting virus into the mesencephalic region of the developing neural tube of embryonic day 2, stage 9–11 embryos [56] with the goal of flooding the developing optic vesicles with virus.

3.3 Injection procedure

3.3.1 Preparation and filling of glass needles—Before beginning, set up the injection area (Fig 1A). Pull several glass needles with tip diameters of ~40 µm using 7 inch Drummond glass capillary tubing (#3-000-203-G/XL) and a Model P-87 Flaming/Brown micropipette puller (Sutter Instruments) with the following settings: Heat 725, Velocity 70, Time 150, and Pull 50. To prepare a glass needle for backfilling with virus, the needle must first be filled with light mineral oil using a syringe fitted with a 27 gauge, 1.25 inch needle if using an oil-displacement injector such as the Nanoject II. To backfill the glass needle with oil it may be necessary to break off the tip of the needle. The goal is to be able to fill the glass needle with oil avoiding producing air bubbles when moderate pressure is applied to the plunger of the syringe containing oil (Fig 1B). We have found that breaking the glass needle at the point where the glass easily flexes when pressure is applied with a forcep usually produces a needle with good flow qualities. It is better to be conservative and remove small pieces of the needle and test oil flow after each piece is removed rather than break off too much. Mount the oil-filled needle into the Nanoject II needle holder and attach the holder assembly to the 3-way micromanipulator. To fill the needle with virus, eject a volume of oil equal to the volume of virus that will be loaded from the needle using the Nanoject controller. Then, without introducing air, pull the virus solution into the needle using the Nanoject controller. Because delivery of virus using the Nanoject II is dependent on physical displacement of the oil in the needle, it is very important that the interfaces between the oil and the rubber gasket in the needle holder and the oil and the virus solution are free of air. To prepare the virus for loading into the needle, thaw an aliquot of virus on ice and add 0.5 µl 0.03% fast green dye to the virus aliquot. Gently mix and pipet the volume of virus to be loaded into the needle onto a sheet of Parafilm™ that has been affixed to a frozen ice pack (Fig 1C). Cover the virus with a few drops of mineral oil to prevent it from evaporating while loading it into the glass needle. In general, we load the needle with sufficient virus to inject 5–6 embryos. During the virus loading procedure, if too much

resistance is encountered creating air pockets between the virus and oil interface, the end of the needle should be opened further by breaking off a small portion of the tip.

3.3.2 Injection of the embryo

1. Candle the eggs to identify those that contain developing embryos. The embryos should be positioned directly under the air cell located at the large end of the egg.
2. Cut a 5–7 mm sided square into the egg shell at the large end of the egg using an X-Acto extra fine saw blade (Fig 1D, E). Gently lift the shell away from the underlying shell membranes. If the underlying membranes are attached to the shell, they can be removed with the shell. To expedite injection, we often open and then reseal several eggs with Parafilm™ (step 5 below) and return them to the incubator until injection
3. Gently remove the shell membranes to access the air cell. The developing embryo may be visible through the chorio-allantoic membrane, but we find that it is usually necessary to peel back a small section of this membrane to visualize stage 8–10 embryos (Fig 1F). To improve visualization of the embryo when first learning this procedure, it is possible to either inject non-toxic dye beneath the embryo or use blue daylight correction filters on the light sources used to eliminate the egg.
4. Once the embryo is located, the needle is positioned for delivery of virus to the neural tube and ~ 0.5 µl of virus is injected. If successful, virus will flow through the neuropore if it has not yet closed and it will be possible to see the virus fill the neural tube (Fig 1G).
5. Following injection, cover the opening in the egg shell with a small square of Parafilm™. The square should only be slightly larger than the hole to minimize its effects on the ability of air to move through the shell. Completely seal the edge of the Parafilm™ to the shell using a warmed spatula (Fig 1H). Use of Parafilm™ to seal the egg prevents desiccation of the egg during incubation and permits visualization of the developing embryo. It is our experience that injected embryos are more likely to develop and hatch if the hole that is created in the shell to inject the embryo is as small as possible.

3.4 Incubation and hatching of injected embryos

Post-injection eggs are incubated large end up at 37.5 °C and 60% humidity. The eggs are slowly rocked three times per day (Sportsman Incubator; GQF Manufacturing; <http://www.gqfmfg.com>) until the embryos reach the desired growth stage. If hatching is desired, the eggs should be moved to a non-rocking basket within the incubator when the chick has begun to pip the egg shell. Several variables can affect hatching; however, we have found that the humidity level in the incubator is a key factor in determining hatching success. For best results, the humidity should be maintained at ~68% for the final two days of incubation. If the incubator is too humid, water will not evaporate from the egg and the amount of air available to the chick in the egg will be insufficient leading to death of the chick. If the humidity is too low, the shell membranes will stick to the chick and the chick will be unable to move in the egg and will be unable to complete the hatching process.

4. Concluding remarks

The development of increasingly safe lentiviral vectors has prompted more investigators to consider using these vectors to deliver transgenes to their cells or tissues of interest. Our most recent research efforts have focused on designing multicistronic transgenes for lentivectors that reliably express two or more proteins in infected cells. We have used IRES sequences, multiple internal promoters, and 2A cleavage peptides to try to achieve this goal. We found that transgenes carrying multiple promoters can express multiple proteins reliably, but use of this approach is limited by availability of suitable promoters that possess desirable expression characteristics and do not interact with each other in the context of the transgene. Promoter issues are not a problem when using 2A peptides. Our data suggest that using 2A cleavage peptides to create multicistronic lentiviral transgenes is currently the most expeditious method to deliver and express multiple proteins in infected cells. Again, we refer readers interested in using 2A cleavage peptides to a recent series of papers that include detailed protocols for developing and confirming the performance of transgenes carrying 2A cleavage peptides.

The primary focus of our research program over the last 25 years has been to develop gene-based therapies for treatment of inherited photoreceptor disease. Our primary model system, the GUCY1*B chicken model of Leber congenital amaurosis – type 1, represents one of only a few avian models of human retinal disease and as such, necessitated development of new vectors and approaches that would allow testing of potential therapies for this disease. While our work has focused on retinal disease, it has not been lost on us that the methods we have developed to introduce transgenes into developing chicken retina would also be of interest to investigators studying neural development. The transduction characteristics and cargo capacities of lentiviral vectors make them ideal vectors for manipulating gene expression in developing tissues. The ability of these vectors to carry relatively large transgenes makes them ideally suited for experiments requiring delivery of multiple proteins and regulatory elements to control their expression.

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Highlights

- Expression characteristics of bicistronic lentiviral vectors in neural retina.
- Detailed method to package lentiviral vectors into virus.
- *In ovo* injection method to infect developing chick nervous system.
- Discussion of incubation and hatching of injected embryos.

Figure 1. Injection of chicken embryos with lentivirus

A. Setup for injection. 1-Nanoject II; 2-dissecting microscope and flexible dual light-pipe high intensity light source; 3-foam egg holder with gauze covering; 4-3-way micromanipulator. **B.** Preparation of tip of glass needle. Fine-tipped forceps are used to break off the tip of the pulled needle (arrow). This step is done before loading the needle with oil and also can be repeated if the needle becomes clogged during injections. **C.** Backfilling needle with virus. The desired volume of virus is pipetted onto Parafilm™ affixed to a cold-pack and covered with mineral oil. The tip of the needle is inserted into the virus and drawn up into the needle (arrow). **D.** Creation of hole in egg shell. A square hole with ~5–7 mm sides is created in the large end of the egg using a fine-toothed hand saw. **E.** Removal of shell using a fine-tipped forcep. **F.** Removal of chorio-allantoic membrane enabling visualization of the embryo (arrow). **G.** Injection of virus containing 0.03% Fast green dye into the anterior region of the developing neural tube of a stage 10–11 embryo. Virus can be seen to exit the neuropore (arrow), outlining the developing head of the embryo. **H.** Sealing the injected egg. Following injection a small square of Parafilm™ is affixed to the shell using a warmed spatula.

Table 1

Cellular Activity of Various Promoters in Chicken Retina

Promoter	Gene	Source of Promoter	Lentiviral Transgenes	Retinal Cells Expressing Transgene ^a	Reference
IRBP156	Interphotoreceptor retinoid binding protein	mouse	IRBP156-tdTOM	INL,R,C	[10]
IRBP1783	Interphotoreceptor retinoid binding protein	mouse	IRBP1783-tdTOM	C	[9]
XOPS1.3	Rhodopsin	frog	XOPS-tdTOM	R	[10]
MOP500	Rhodopsin	mouse	MOPS-GFP	R	[9]
GCAP292	Guanylate cyclase activating protein-1	chicken	GCAP292-GFP GCAP292-nlacZ	INL,C	[9] [8]
GCAP1436	Guanylate cyclase activating protein-1	chicken	GCAP1436-nlacZ	INL,P	[8]
GCAP4009	Guanylate cyclase activating protein-1	chicken	GCAP4009-nlacZ	P	[8]
RK	Rhodopsin kinase	human	RK-GFP	R,C	[10]
GCE7	Guanylate cyclase-1	human	GCE7-nlacZ	P	[57]
GCE8	Guanylate cyclase-1	human	GCE8-nlacZ	P	[57]
GCE1	Guanylate cyclase-1	human	GCE1-nlacZ	INL,P	[57]
NRLL	Neural retina leucine zipper	mouse	NRLL-tdTOM	R,C	[10]
CD44	Cluster differentiation	mouse	CD44-GFP	G,M,INL,P	[10,58]
VIM	Vimentin	mouse	VIM-GFP	M,INL,P	[10,58]
GFAP	Glial fibrillary acidic protein	mouse	GFAP-GFP	M	[10,58]

^aINL-inner nuclear layer; R-rod; C-cone; P-photoreceptor; G-ganglion cell; M-Muller cell

Table 2

Activities of Dual Promoter Lentivectors in Chicken Retina

Transgene Construct	Upstream Expression ^a	Downstream Expression ^a	Dominant Expression	% Cells Expressing Both ^b	Reference
GCAP292-GFP-IRBP1783-tdTomato	C	C	Upstream	Variable	[9]
IRBP1783-tdTomato-GCAP292-GFP	C	C	Upstream	Variable	[9]
IRBP1783-tdTomato-MOP500-GFP	C	R	ND	ND	[9]
EF1α-tdTomato-GCAP292-GFP	Ubiquitous	C	Upstream	Low	[9]
RK-GFP-IRBP156-CHER	P	P	Downstream	Low	[10]
IRBP156-CHER-RK-GFP	P	P	Upstream	High	[10]
XOPS-tdTOM-MOPS-GFP	R	R	Equal	Medium	[10]
MOPS-GFP-XOPS-tdTOM	R	R	Downstream	High	[10]
CD44-CHER-VIM-GFP	ND	M	Downstream	ND	[10]
RK-GFP-RK-CHER	P	P	Downstream	Low	[10]
EF1-GFP-EF1-mCherH	Ubiquitous	Ubiquitous	Downstream	Low	[32]
EF1-GFP-WPRE-EF1-mCher	Ubiquitous	Ubiquitous	Equal	Low	[32]

^aINL-inner nuclear layer; R-rod; C-cone; P-photoreceptor; G-ganglion cell; M-Müller cell; ND-Not detectable

^bLow: 0–30%; Medium: 31–60%; High: >60%; Variable: No average percentage possible

Table 3

Activities of Bicistronic 2A Lentivectors in Chicken Retina

Transgene Construct	Expression Region	Cleavage Efficiency ^a	Dominant Expression	% Cells Expressing Both ^a	Reference
EF1-GFP-2A-mCherH-WPRE	Ubiquitous	High	Equal	High	[32]
EF1-GFP-Furin-2A-mCher-WPRE	Ubiquitous	ND	ND	ND	[32]
EF1-GFP-2A-GC1-WPRE	Ubiquitous	High	Equal	High	[32]

^aLow: 0–30%; Medium: 31–60%; High: >60%; Variable: No average percentage possible; ND: Not detectable