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DNA delivery in adult mouse eyes: An update with corneal endothelium outcomes

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Abstract

Ocular injection (intravitreal, subretinal, or into the anterior space) is an efficient approach to deliver many classes of drugs, cells, and other treatments to various cell types of the eye. In particular, subretinal injection is efficient since delivered agents accumulate as there is no dilution due to transport processes or diffusion and because the volume of the interphotoreceptor space (IPS) is minimal (10–20 microliters in the human eye, less than 1 microliter in the mouse eye). We previously reported methods using subretinal injection and electroporation to deliver DNA to photoreceptor and retinal pigment epithelium (RPE) cells in retinas of live mice(1–3). Here we detail further optimization of that approach and additionally report its use in delivering DNA expression plasmids to the corneal endothelium.

Keywords

subretinal; injection; electroporation; interphotoreceptor space; transfection; dilation; sclera; cornea; reporter gene expression; subretinal bleb; cornea; corneal endothelium

1. Introduction

Gene therapy for some forms of blinding diseases such as Leber Congenital Amaurosis Type 2 (LCA 2) has progressed into advanced clinical trials (ref). These treatments require subretinal delivery of DNA constructs. The surgeries are complicated and additionally rely on viral-mediated transfection. Thus, continued development of DNA delivery to the subretinal space is warranted. Similarly, DNA delivery to ocular anterior segment targets such as the corneal endothelium, are still evolving (4–8).

Separately from clinical considerations, optimizing ocular gene delivery for the mouse is critical. Genetics and genomic modifications in the mouse are highly informative, making the mouse indispensable in much of biomedical research. This is particularly so for vision research as it is the smallest mammal that has an eye resembling that of humans. However, the small size of the eye and the relatively large size of the lens make subretinal surgery difficult in mice.

To address these needs and take advantage of animal models, several groups have developed techniques for gene delivery to the eye. We previously reported methods using subretinal injection and electroporation to deliver DNA to photoreceptor and retinal pigment epithelium (RPE) cells in retinas of live mice(1–3). Here we detail further optimization of that approach and additionally report its use in delivering DNA expression plasmids to the corneal endothelium.

Several injection approaches are used for delivery of drugs, DNA constructs, and other agents to the subretinal space, including a transscleral-vitreous-transretinal route and a transscleral-transchoroidal-Bruch's membrane approach that does not penetrate the retina (9, 10, 11, 12). Though effective, the small size of the mouse eye and the comparative toughness of the sclera increase the risk of hemorrhages at the ciliary body or choroid that cause autofluorescence and retinal damage, preventing further treatment or experimentation. To avoid this, we adapted the transcorneal approach originally developed by Timmers and coworkers for rat subretinal injections (13).

Of the several approaches available for transfecting DNA into cells (reviewed (2)), viral packaging and electroporation are the most efficient, with virus-mediated delivery providing 100% transduction efficiency (14) and electroporation providing 90% efficiency (15) in cultured photoreceptor cells.

We use electroporation to transfect ocular cells following injection as it is inexpensive, safe, and easy to replicate if done under well-controlled conditions (15–17). Here we update our electroporation protocol that produces abundant reporter gene expression from plasmids in the RPE of the living adult mouse (1, 2). We additionally show that the approach results in reporter gene expression in corneal epithelial cells.

2. Materials

2.1 Mice

1. Strains: We have used pigmented and nonpigmented mice aged between birth and 9 months old at time of surgery. We do not think that there is a need to modify the protocol for older mice, but we have not tested mice older than 10 months. For neonatal mice, the procedure of Price et al. (9) is fast and easy.
2. Mouse husbandry: Mice should be used according to regulatory agency guidelines and must be approved by a local Institutional Animal Care and Use Committee. For instance, in our work, all procedures were approved by the Emory University Institutional Animal Care and Use Committee and conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed at 22 °C in facilities managed by the Emory University Division of Animal Resources and given standard mouse chow (Lab Diet 5001; PMI Nutrition Inc., LLC, Brentwood, MO) and water ad libitum. They were maintained on a 12 h:12 h light-dark cycle, with daytime lighting ranging 200–750 lux outside the cage depending on lower, middle, or top shelf position of the cage rack. We have found it useful to monitor light

levels, temperature, and humidity with a datalogger. This can identify unexpected changes in lighting, humidity, or temperature that can alter the outcomes of any in vivo study.

2.2. Reporter gene plasmid

The reporter gene plasmid, pVAX-tdTomato, a gift from Dr. Ton N.M. Schumacher of the Department of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands, was used throughout these studies. The plasmid contains the CMV Immediate Early promoter driving expression of tdTomato, a bovine growth hormone poly(A) signal on the 3' flanking side of the tdTomato cDNA, and the Kanamycin resistance gene for selection and growth (18). Plasmid was isolated from transformed DAM⁻/DCM⁻ cells (C2925H; New England Biolabs) after overnight growth in Luria broth using a GIGAprep kit (Qiagen) following the manufacturer's protocol. Plasmid was dried using a speedvac system and pellets stored at -20 °C.

2.3. Equipment

1. Dissecting microscope system: Olympus SZX2-ZB16 stereo microscope (Hunt Optics; Pittsburgh, PA), The light source is a halogen lamp mounted to an epifluorescence adapter to provide true coaxial illumination through the objective lens, which is far better than a ring light for illuminating the back of the mouse eye, critical for the subretinal injection of pigmented mice.
2. Video recording: Videos are recorded using a Panasonic GPUS932HT HD Video camera (Hunt Optics and Imaging) interfaced to a KONA LHe HD-video capture card (AJA Video Systems, Grass Valley, CA) in a Mac Pro (Apple Computer, Cupertino, CA). Videos were edited with Final Cut Pro (version 6; Apple Computer).
3. Injection equipment: NanoFilTM Sub-microliter injection system with a UMP-II microsyringe pump and Micro4 controller with a footswitch (World Precision Instruments; WPI, Sarasota, FL).
4. Mouse warming equipment: Two systems are used to maintain the mouse at 37 °C. One is a commercial product, a T/Pump TP500; Gaymar, Orchard Park, NY. The other is an aluminum block we cut channels in for circulating water warmed by a Lauda Circulating water bath pump. See note 3.
5. Beveled 34 gauge needles (catalog number NF34BV-2), blunt 35 gauge needles (catalog number NF35BL-2), and curved forceps (catalog number 15915) were obtained from WPI. Most other incidental equipment and tools were from Fisher Scientific (Pittsburgh, PA) or VWR (West Chester, PA). See Note 2.

³For recovery, mice are placed into a cage with a paper towel under to keep bedding from sticking to eyes. The cage is half on and half off a heating pad. Mice recovering from anesthesia are placed on the heating pad side of the cage. Once mice are ambulatory, they thermoregulate and can move to the unheated side of the cage. This prevents overheating.

²Needles should be replaced in advance of their becoming dull to ensure smooth puncture of the cornea. Corneal tearing often indicates that the beveled needle needs to be replaced.

6. The electroporator was a commercial square wave generator (BTX model ECM830; Harvard Apparatus, Holliston, MA). Others are acceptable, too.
7. Platinum-iridium 20 gauge wire (catalog number 50822164; Fisher).
8. Small test-jumper leads (catalog number 278-001; Radio Shack Corporation, Fort Worth, TX).
9. Optispears (Ocusoft, Inc., Richmond, TX)
10. Microknife. (Blade size 15° stab knife straight, Sharpnpoint, catalog number REF72-1501, Reading, PA)
11. A Rodent Restrainer (Vac-Tec, Inc., Euless, TX).

2.4 Pharmaceuticals and other surgical compounds

1. Pre-mixed 80mg/ml and 12mg/ml Ketamine and Xylazine solution (K-113; Sigma-Aldrich, St. Louis MO).
2. Tropicamide Ophthalmic Solution USP, 1% (10 mg) from Bausch & Lomb
3. Atropine Sulfate Ophthalmic Solution USP, 1% (10 mg) from Bausch & Lomb
4. Proparacaine hydrochloride ophthalmic solution USP, 0.5%; National Drug Code (NDC) 17478-263-12; Akorn Inc., Buffalo Grove, IL.
5. Vidisic^R Augengel (catalog number 1-19006, distributed by Dr. Mann Pharma, Berlin, Germany) is a transparent clear ocular hydro-gel, which contains high molecular weight polyacrylic acid. It was the kind gift of Dr. Philipp Lirk, Department of Anesthesiology and Critical Care Medicine, Medical University Innsbruck, Austria. Other clear ophthalmic grade hydrogels, including viscous methylcelluloses are acceptable as well.
6. "Refresh" artificial tears eye drops (Allergan, Irvine, CA).
7. Triple antibiotic ointment (Taro Pharmaceuticals, Inc., Hawthorne, NY), which contains bacitracin, neomycin sulfate, and polymyxin B.

3. Methods

3.1. Worksheet

A record of the subretinal injection procedure should be kept. Documentation should include ear tag number (ear tags from WPI), date of birth, sex, amount of anesthesia, bleb size (small, medium, or large), and, especially important, the number of blebs. Also record any complications including backflow through the retinotomy, hemorrhage, lens damage, corneal clouding, or the presence of air bubbles. A qualitative comment on the outcome of each injection is encouraged.

3.2. Injection material preparation

Resuspend plasmid DNA (pVAX-TdTomato) in sterile water at 2 mg/ml. The marker dye Fast Green (catalog number BP123-10, Fisher, Fairlawn, NJ) is included in all injected

solutions at 0.1% (W/V). Centrifuge the plasmid solution at $10,000 \times g$ for 5 min to sediment any particulates from the solution that might clog a 35 gauge needle (see Note 1). Do this immediately before loading the needle and injection syringe.

3.3. Surgical sterility

Use sterile surgical technique throughout. Sterilize fluid lines, surgical instruments, and needles by repeated rinsing with 70% ethanol and sterile water. Start with water to unclog any residual materials in equipment. Switch to 70% ethanol and then back to water. After these washes, flush once or twice with delivery solution (e.g., DNA plasmid in buffer, etc.) followed by filling syringe again with delivery solution (see Note 1).

3.4. Anesthesia and pupillary dilation

1. Perform injections between 8:00 AM and 6:00 PM (i.e., during lights-on of the photoperiod). Keep each mouse on a 37 °C pad during and after surgery until it regains consciousness and mobility (see Note 3). Mice are anesthetized with 80 mg ketamine and 12 mg xylazine per kilogram body weight (K-113; Sigma-Aldrich, St. Louis, MO) and achieve adequate anesthesia in 3 to 5 min.

2. Ketamine/Xylazine preparation: To make a working solution, dilute the stock 1:5 with sterile dPBS. Keep the working solution ice cold at all times. The working concentration is 16 µg/µl Ketamine and 2.4 µg/µl Xylazine. So, for a 20 g mouse, 100 µl are injected into the hind leg muscle or I.P. with a 29 gauge needle. Be sure to record these drugs accurately in a suitable logbook following DEA regulations.

Anesthetize the cornea topically with one drop of proparacaine for 2 min. Remove excess with an optispear. Add a drop of Refresh artificial tears on the eye to prevent drying, and remove excess.

For albino mice, put one drop of tropicamide onto each cornea and wait about 3 min for pupils to dilate. For pigmented mice, place one drop of atropine onto each cornea and wait about 6 min for dilation. In this waiting period, place the mouse on a heating pad and cover the mouse to shield from light to help dilate the pupils (a cardboard boxtop covering the entire mouse that does not impede airflow is adequate; see Note 4)). If not dilated at the end of the waiting period, apply another drop of dilatory drug.

3.5 Subretinal injection

Perform injections between 8:00 AM and 6:00 PM (i.e., during lights-on of the photoperiod). When moving to the injection scope, remove excess dilatory drug with an Optispear. Mice should be monitored carefully for signs of pain and distress during and after

¹For clogging issues, if a drop of water is seen at the junction of the tubing and syringe or between needle and connector piece, then apparatus is clogged. Repeated rinses with water will usually unclog most pieces except the needle. The blunt needle often must be replaced due to clogging. To unclog the blunt needle, put the needle into syringe backwards (the tip of the blunt needle is thinner than the other end. It is easier to get such "stuff" out of the bigger end) to repeatedly push sterile water through until the needle is unclogged. Just pushing sterile water through usually does not unclog well. Ethanol should not be used to flush a clogged needle. Sonication can be used to unclog needles, too.

⁴Keep the mouse out of light as much as possible prior to surgery and after dilation to keep pupil from constricting. As experience is built and the overall procedure is completed in less time, this consideration becomes less important.

surgery. Keep each mouse on a 37 °C pad during and after surgery until it regains consciousness and mobility (see Note 3).

Position the mouse with its nose pointing away from the surgeon and its left eye facing up toward the light and the microscope. Place a drop of hydrogel (Vidisc^R) on the mouse cornea. Grasp the corner of a 22×40 microscope coverslip by hand and adjust the coverslip on the Vidisc^R eye gel in such a way that the fundus, its blood vessels, and the optic nerve head can be seen. Do not press down on the cover slip. It should just rest on the eye. Verify that the pupil is fully dilated. This fundus exam serves to assess the condition of the eye before injection and as a comparison for the postoperative condition of the retina. Note the structure and appearance of blood vessels and optic disc. (For pigmented animals, the lighting should be about 54,000 lux, and for nonpigmented the light source should be about 4800 lux (adjust with neutral density filters to reduce the light level, or use another source).

Adjust the position the mouse on a heating pad as necessary for the surgical procedure. It is not necessary or desirable to restrain the mouse. Remove excess Vidisc with an Optispear. Apply Betadine (5%, ophthalmic grade, Alcon, NDC 0065 0411 30; Ft. Worth TX) to eye, lids, and fur surrounding eye. Remove excess with an Optispear and additionally apply drops of Refresh artificial tears to rinse excess betadine from eye and surrounding skin and to keep eyes moist. At this point, it should take about 90 seconds to complete the subretinal injection. Grasp the left eye with curved forceps held in the surgeon's non-dominant hand so that the eye is slightly proptosed by partially and gently closing the forceps. With a 34 gauge beveled needle (held in the surgeon's dominant hand), lance the cornea superiorly near the limbus penetrating into the anterior chamber at an oblique (nearly tangential) angle. The tip of the needle should be pointed downwards to avoid nicking the lens. The point of the bevel should always be down and the opening of the needle up. Some stretching of the cornea is advantageous, allowing the wound to effectively reseal itself at the end of surgery. It is important to have a good grip on the eye and that the insertion of the needle is done in a firm but smooth motion so that the eye is punctured cleanly and not displaced from the grip of the forceps. Remove the beveled needle. Replace it with a blunt 35 gauge needle connected to the injection system (WPI). Advance the needle into the anterior chamber until the tip is centered on the optical axis. With a sweeping motion, move the tip of the needle through the pupil, around the lens, and into the vitreous. While rare, if the lens is nicked, the surgery should be abandoned.

Advance the needle tip to puncture the retina. The lens magnifies the view of the needle. Once you encounter a slight resistance to the needle, STOP! You have reached the RPE layer. A gentle amount of pressure (the touch of which must be learned by experience) is applied to penetrate the neural retina into the subretinal space, but not so much that the tip penetrates or damages the RPE sheet. The motion of the needle is a sweeping motion downwards (which is nasally based on how the mouse is positioned). There is no difference in feel of resistance as the needle sweeps through the vitreous and then the neural retina and then as the tip touches the RPE/choroid. However, applying too much force during the sweep will lead to the tip entering the choroid and causing bleeding.

Being careful of the slightest of movement, use the foot pedal to inject fluid into the subretinal space. The nanojector system should be set to deliver 1000 nl at a rate of 170 nl/sec. It can be useful for an assistant to press the injection button on the face of the nanojector, but the foot pedal is just as easy. The delivery of the correct volume will result in visible blebs at or near the injection site. Blood in the fundus means that the tip pressure was too great and the choroid was damaged. Too little pressure and the retina is not penetrated. Marker dye in the vitreous indicates the pressure was insufficient and the needle did not enter the subretinal space.

Leave the injection needle in the subretinal space for a few seconds (no more than 10 s) to allow pressure in the injection system to equilibrate with subretinal bleb pressure, otherwise large fractions of the subretinal injection material will leak into the vitreous. Pull the needle out slightly, wait another second, and then remove the needle entirely. Remove the needle slowly to allow the hole in the retina to reseal and to avoid damaging the lens, iris, and corneal endothelium during removal.

After surgery, place more hydrogel on the cornea to examine the fundus. A critical step is to count the number of large blebs on the fundus. Complete the post-injection exam to confirm the presence of the blebs. Place triple antibiotic ointment. Verify the ear tag.

3.6. Electroporation

Eyes are electroporated immediately following subretinal injection. Make electrodes by wrapping 20 gauge platinum-iridium wire around a sharpened pencil tip, creating a 1.5 to 2 mm loop. Clip the loops to jumper leads and then to the BTX electroporator. Restrain the mouse to avoid movement during this procedure. Position one platinum loop electrode (anode) directly underneath the retina bleb site on the scleral surface of the mouse globe, and the other loop (cathode) should be positioned diametrically opposite from the retinotomy. Do not place anode too far posteriorly. When current is applied through the electrode, the mouse's head will move backwards. If the anode is placed too posterior on the eye, the eye will not be able to move with the head and the optic nerve will be damaged. Conversely, do not place the anode too anterior or it will contact the cathode, causing a short circuit that damages tissue. Thus, space the electrodes 1.5 to 2 mm apart. Try to be as consistent as possible in this spacing, as it determines the potential difference per cm, one of the critical variables in consistent transfection efficiency. Press the electroporator foot pedal to apply current.

Optimal conditions and minimum requirements ought to be investigated by varying the voltage, pulse length, number of pulses, and number of pulse trains. With our apparatus, an optimum was found with 80 V, 5 pulses, 5 ms pulse duration, 1 s interval between pulses, and two pulse trains. The range of conditions that we tested were: 0.1 ms to 100 ms for pulse length, 0 to 200 V for potential difference, 5 to 20 pulses, 0.125 and 1 s interval between pulses, and one or two pulse trains. In some instances greater transfection was obtained by repeating the electroporation after electrodes had been repositioned to approximately perpendicular to the first current application.

Typical negative controls include omitting plasmid (vehicle-only subretinal injection) or omitting electroporation in different mice. The contralateral eye served as an uninjected control in all mice.

3.7. Recovery

Cover the animal on a 37 °C heating pad until it is awake and actively moving. Transfer it to a clean cage by itself for 1 to 2 h. Apply more Refresh every 10 minutes to keep corneas from drying out. Afterwards, return the mouse to its home cage until analysis, usually several days after treatment. After experimentation and analysis, mice are euthanized by CO₂ asphyxiation.

3.8. Outcome measures of safety

For RPE cell sheets, TUNEL staining as a marker of apoptosis is an effective tool to assess damage. Flatmounts are created (19). Essentially, the eyecup flatmount includes all the cornea and sclera, but the neural retina, iris, ciliary body, and lens were removed (20, 21). A puncture was made in the cornea with a microknife, and iridectomy scissors (WPI) were used to make four radial cuts, starting at the center of the cornea, and extending toward the optic nerve. The flattened eyecups were placed on microscope slides in 100 µl of dPBS. Primary and secondary antibody staining solutions were pipetted on and off with handheld pipettors, but otherwise did not differ from standard immunostaining procedures. They are mounted in Vectashield hardset, and ought to be examined as soon as the hardset has fully solidified. We normally allow the mountant to set overnight and image the slides the next day. Tissue death under optimized conditions should be <1% of RPE cells. An internal positive control for cell death is apoptosis in the corneal endothelium.

3.9. Outcome measures of efficacy

1. Injection efficacy: Determining the whether an injection was successful requires a thorough examination of the fundus after surgery. Three to five blebs underlying the neural retina is a sign of proper inflation of the subretinal space. These blebs are clearly visible, and they demonstrate that the injected material was well confined within the subretinal space. We have not observed any cases of more than three blebs in nonpigmented mice. Eyes with only one or two blebs were usually accompanied by evidence of a torn or damaged retina, as seen on fundusoscopic examination. Nonpigmented mice without three blebs were excluded from experimental test groups.

The tdTomato reporter gene has an excitation optimum at 554 nm, and a emission maximum at 581 nm. Compared to other naturally fluorescent proteins, tdTomato has reduced photobleaching and provides excellent fluorescence (22). TdTomato was excited using 561 nm laser line and emissions were filtered using a 605/75 bandpass filter. About 30–40% of RPE cells should express the reporter gene, in this case the red fluorescence from TdTomato (Figure 1). The polygonal pattern of RPE cells is observed in both transfected and nontransfected cells, and there is no characteristic bias in the transfection of cells of lesser or greater polygonality.

The injection technique is successful in our hands about 80% of the time. It requires a significant amount of time to learn. Depending on the amount of practice and prior surgical skills, we find it takes 50–100 surgeries to become proficient in the subretinal injection of the adult mouse eye. This is in contrast to the relative ease in the subretinal injection of neonatal rat pups (13). We found the outcome of the surgery could be rapidly determined postoperatively by examining the mouse fundus. The subretinal injection raised blebs, indicating that the retina was elevated from the RPE sheet, and the persistence of these blebs demonstrated the retinal hole had sealed. No bleb meant that the surgery had failed, and the mouse should not be used for further experimentation. Initially, we expected only one large bleb, because that is what has traditionally been observed in subretinal injections of neonatal rat pups. However, we found the best outcomes were when there were three blebs in nonpigmented mice and four blebs in pigmented mice, and when the perimeters of these blebs, as viewed by fundoscopy, were demarcated by major blood vessels. It should be noted that bleb formation is also used as a sign for successful subretinal injection in human patients (23–26).

2. Electroporation efficacy: Assessment of the efficacy of electroporation is more subtle. After electroporation, tdTomato fluorescence should be found in a tight patch of RPE cells nearest to the positive electrode (anode) (Figure 1). No fluorescence was detected at the negative electrode (cathode), which is nearer to the cornea. Accurate placement of the anode circumscribing the bleb and highly concentrated plasmid are necessary to achieve delivery of the plasmid into RPE cells and expression of tdTomato. Under suboptimal conditions, tdTomato fluorescence was detected in the cornea, ciliary body, and iris. This result occurred with much longer pulse durations (25 to 50 ms). Voltages beyond about 100 V resulted in immediate evidence of burn damage and were not tested further. Pulse lengths were varied from 1 ms to 50 ms at different voltages. At 10 ms or longer, the area of the flatmount that showed evidence of transfection extended beyond the immediate location of the electrodes as far as the cornea.

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Figure 1. A typical result of subretinal injection and electroporation of the mouse eye
Transfection and expression of TdTomato were detected two days after injection. Red represents TdTomato protein fluorescence and blue represents actin rings stained with AlexaFluor688-phalloidin that mark the outline of the RPE cells. Both red hexagons and blue hexagons are readily detected in abundance near the injection site. The brighter blue bands at the outer edges of the retina petals are ciliary body. Beyond these are the corneal flaps showing.

Figure 2. Effect of subretinal injection and electroporation of the mouse eye on corneal endothelium

The brighter blue bands are ciliary body. Transfection and expression of TdTomato were detected two days after injection. Red represents TdTomato protein fluorescence and blue represents actin rings stained with AlexaFluor688-phalloidin that mark the outline of corneal epithelial cells. Both red and blue cells are readily detected in abundance near the injection site.

Table 1

Troubleshooting guide for subretinal injections

Problem	Probable cause	Solutions
Lens cloudy	Lens capsule nicked during surgery	Avoid the lens by sweeping needle around it
No blebs	Not penetrating the retina	Ensure that the needle goes to the back of the eye, possibly noting this by touch; press a little harder
No blebs	Torn retina or retina hole; fluid leaks out quickly into vitreous	Penetrate retina in a single motion
No blebs	Fluid leaks out quickly into vitreous or a poorly inflated bleb	Pause for 5–10 s before removing the blunt needle from the subretinal bleb. This allows pressure equilibration. Also, observation of blebs depends on accurate aiming of the camera at the area expected to contain blebs
No Blebs	Clogged needle/no fluid injected	Check for fluid delivery
Blood	Pressing the needle against iris	Ensure that pupil is completely dilated
Blood	Penetrating into the choroid	Press gently
Blood	Nicking the ciliary body	Sweep closer to lens
Cloudy cornea	Eye was not kept moist before surgery	Apply lubricating eye drops between Proparacaine and phenylephrine application
Air bubbles	Air in lines or solutions not degassed	Degas solutions. Flush the lines and prime them with water before filling with delivery solution