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Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs

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Cover Letter

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To: *Journal of Visualized Experiments (JoVE)*

From: Negin P. Martin, Ph.D.

Subject: Manuscript Submission

April 20th, 2018

Dear editorial board,

Please find attached our manuscript entitled " Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs" for consideration to publish in *JoVE*. We were very excited to be invited by Dr. Indrani Mukherjee to submit a manuscript.

In this manuscript, we validate an effective and original method for delivering genes to mouse fertilized eggs by combining laser-perforation of zona pellucida and lentiviral gene delivery. Multiple departments at our Institute combined experiences to develop this method for gene delivery to mouse fertilized eggs. This method has been received very well by mouse geneticist as an alternate method for spatiotemporal genetic manipulation of mouse embryos. In this protocol, instead of delivering genes to mouse fertilized eggs one at a time utilizing pronuclear microinjection, the gene(s) of interest are delivered rapidly and in bulk without micromanipulation. We demonstrate that XYClone laser perforation of zona which is typically used for *in vitro* fertilization, also provides a venue for lentiviruses to diffuse across the zona and to infect mouse embryos. We provide a detailed protocol for successful gene delivery in our manuscript. Once the zona barrier is permeablized by the XYClone laser, it makes embryos accessible for gene delivery via various types of viruses. Thus, providing a powerful tool for delivering genes to control early stage cell fate, to influence the differentiation of embryonic stem cells, and to create transgenic, knockdown or knockout animal models. We believe that our findings will generate broad interest in your readership and hope that our paper merits publication in *JoVE*.

We will be happy with selection of any member of the *JoVE* editorial board for review. I would like to thank you in advance for considering our manuscript. Should you have any questions or concerns please feel free to contact me.

Sincerely yours,

Negin P. Martin -S Digitally signed by Negin P. Martin -S Date: 2018.04.20 20:27:09 -04'00'

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TITLE:

Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs

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KEYWORDS:

Transgenesis, XYClone laser, lentivirus, mouse fertilized eggs, mouse embryos, gene delivery, transduction

SHORT ABSTRACT:

Mouse fertilized eggs and early stage embryos are protected by the zona pellucida, a glycoprotein matrix that forms a barrier against gene delivery. Here, we describe a protocol for perforating the zona with a laser to transduce embryonic cells with lentiviral vectors and to create transgenic mice.

LONG ABSTRACT:

Lentiviruses are efficient vectors for gene delivery to mammalian cells. Following transduction, the lentiviral genome is stably incorporated into the host chromosome and is passed on to progeny. Thus, they are ideal vectors for creation of stable cell lines, *in vivo* delivery of indicators, and transduction of single cell fertilized eggs to create transgenic animals. However, mouse fertilized eggs and early stage embryos are protected by the zona pellucida, a glycoprotein matrix that forms a barrier against lentiviral gene delivery. Lentiviruses are too large to penetrate the zona and are typically delivered by microinjection of viral particles into the perivitelline cavity, the space between the zona and the embryonic cells. The requirement for highly skilled technologists and specialized equipment has minimized the use of lentiviruses for gene delivery to mouse embryos. Here, we describe a protocol for permeabilizing the mouse fertilized eggs by perforating the zona with a laser. Laser-perforation does not result in any damage to embryos and allows lentiviruses to gain access to embryonic cells for gene delivery. Transduced embryos can develop into blastocyst *in vitro,* and if implanted in pseudopregnant mice, develop into transgenic pups. The laser used in this protocol is effective and easy to use. Genes delivered by lentiviruses stably incorporate into mouse embryonic cells and are germline transmittable. This is an alternative method for creation of transgenic mice that requires no micromanipulation and microinjection of fertilized eggs.

INTRODUCTION:

Here, we describe a method for permeabilizing the zona pellucida of mouse fertilized eggs to make embryonic cells accessible for gene delivery by lentiviruses. Lentiviruses are designed by nature for efficient gene delivery to mammalian cells. They infect dividing and non-dividing cells and integrate the lentiviral genome into their host chromosomes[1]. The range of lentiviral host cells is readily expanded by pseudotyping the recombinant lentivirus with the vesicular stomatitis virus glycoprotein (VSV-G), due to the broad tropism of the VSV-G protein[2]. Following transduction, lentiviral genes are stably integrated and expressed as part of their host chromosomes creating an ideal tool for generating transgenic animals. If delivered to early stage embryonic cells, the lentiviral genome is replicated and expressed in the entire organism. Lentiviral transduction has led to the production of mice, rat, chicken, quail and pig[3-7] among other species of transgenics. The typical method of lentiviral gene delivery, however, requires skilled technicians and specialized equipment to overcome the zona pellucida barrier that encapsulates the early stage embryos. The overall goal of this method is to describe how to permeabilize the zona using a laser to facilitate lentiviral gene delivery.

Mammalian eggs are surrounded by the zona pellucida which hardens following fertilization to protect the fertilized eggs against polyspermy and to limit environmental interactions[8,9]. The zona forms a barrier that keeps lentiviruses away from the embryonic cells until the embryos are hatched as a blastocyst. Cultured mouse fertilized eggs hatch after 4 days and must be implanted into pseudopregnant mice prior to hatching for normal development into pups. Therefore, for transduction, lentiviruses are microinjected before hatching from the zona into the perivitelline cavity, the space between the zona and the embryonic cells.

The zona pellucida is often removed for *in vitro* fertilization of human eggs to increase the fertilization rate[10]. However, chemical removal of mouse zona pellucida adversely affects mouse embryo development and is harmful to embryonic cells[11,12]. Other methods for gene delivery to mouse fertilized eggs overcome the zona pellucida barrier by direct microinjection of DNA into the cell nucleus [13]. Pronuclear microinjection is an efficient means of delivering genes to embryos. However, since each embryo is held in place individually for microinjection, the practice can be laborious and time consuming for a novice user.

Other methods such as electroporation and photoporation are useful for transient and short-term gene delivery to mouse fertilized eggs[14-16]. These methods are extensively used for delivering CRISPR-Cas9 components and recombinases. However, electroporation and photoporation delivery of genes cannot be used efficiently to create transgenics. Spermatozoa that are collected from punctured mouse epididymis can also be transduced by lentiviruses and used for *in vitro* fertilization to produce transgenic animals[17-20].

Here, we facilitated the lentiviral gene delivery to mouse embryos by permeabilizing the zona using a laser. The XYClone laser was developed as an aid for *in vitro* fertilization[21] and cultivation of embryonic stem cells[22]. It is a small apparatus that is simple to setup and easy to use. Once installed on a microscope, it occupies the space of an objective lens and the accompanying software allows for aiming the laser while looking through the microscope eyepieces (see PROTOCOL: section 3). Once the zona is perforated by the XYClone laser, lentiviruses can be introduced into the culture media for gene delivery[23]. Multiple lentiviruses could be used to simultaneously deliver several genes for chromosomal incorporation.

This protocol will describe how to isolate and culture mouse fertilized eggs, illustrates the use of laser for perforation of the zona pellucida, and demonstrates the transduction of mouse embryonic cells by lentiviruses.

PROTOCOL:

All animal procedures and treatments used in this protocol were in compliance with the NIH/NIEHS animal care guidelines and were approved by the Animal Care and Use Committee (ACUC) at the NIH/NIEHS, Animal Protocol 2010-0004.

1. Preparations

1.1. Prepare/purchase recombinant non-propagating lentiviruses carrying your gene of interest. In this study, lentivirus SBI511 expressing copepod green fluorescent protein (copGFP; abbreviated to GFP) from an elongation factor 1a promoter was used for transduction. Standard protocols[2] were used to produce and titer lentiviruses at titers higher than 1e8 transducing units per ml (TU/ml).

Note: Use caution and bleach all material that have been in contact with lentiviruses. Refer to your institute's guidelines for safe use and handling of lentiviruses.

1.2. Setup breeding pairs of desired mouse strain the day before harvesting embryos. In this experiment, C57BL/6J strain of mice was used. Female mice used for ovaries and oviduct collection were not treated with any hormones for superovulation.

1.3. 2-24 hours prior to harvesting mouse fertilized eggs, prepare several Potassium Simplex Optimized Medium (KSOM) drop plates as follows: add a 50 µl drop of KSOM medium to the middle of a 35 mm tissue culture-treated dish and cover with 2 ml of Dimethylpolysiloxane (DMPS5X) and place at 37 ^{o}C, 5% CO_2, 5% O_2 and 90% N_2 to equilibrate.

Note: Use disposable sterile plates and discard following exposure to lentiviruses.

1.4. Prepare 1x solution of hyaluronidase in M2 medium from stock solution (100x, 30 mg/ml, store at -20 °C). 100x stock solution was prepared in water.

1.5. 24 hours post laser-assisted transduction of mouse fertilized eggs, set up breeding pairs between vasectomized male and female mice to prepare pseudopregnant female mice.

2. Isolation of Mouse Fertilized Eggs

2.1. 16-24 hours post mating, select female mice with vaginal plugs indicative of successful mating, humanely euthanize, and isolate ovaries and oviducts according to standard protocols[24]. The mice used in this study were euthanized by cervical dislocation under deep CO_2 or isoflurane inhalation.

2.2. Transfer the ovaries and oviducts to a 35 mm dish containing M2 media.

2.3. For each ovary, tear ampulla apart to release fertilized eggs surrounded by cumulus cells.

2.4. Transfer fertilized eggs and cumulus cells to a 35 mm dish containing 1x hyaluronidase in M2 medium and incubate at room temperature for 3-5 minutes.

2.5. Pipet fertilized eggs up and down to release the cumulus cells.

2.6. Transfer fertilized eggs to a 35 mm dish containing M2 media and pipet up and down to wash off hyaluronidase.

2.7. Transfer fertilized eggs to a 35 mm dish containing KSOM and pipet up and down to wash off the remaining M2 media and hyaluronidase.

2.8. Transfer fertilized eggs to the prepared KSOM drop plates from step 1.3.

Note: Mouse fertilized eggs in KSOM must be kept in a tissue culture incubator at 37 °C, 5% CO_2, 5% O_2 and 90% N_2 to equilibrate. Removing plates from the incubator for longer than 15 minutes will result in damage to embryos.

2.9. Allow fertilized eggs to recover for 2 hours in the incubator before moving to the next step.

3. Perforation of Mouse Fertilized Eggs with XYClone Laser

3.1. Setup and calibrate XYClone laser according to the manufacturer's recommendation. Briefly, attach the laser controller box wire to the laser apparatus on the microscope. Attach the laser controller box to the computer running the laser software via a USB port. Plug in the laser controller and switch it on. Looking through the eyepiece, perforate a test sample (e.g. dry-erase markings on a glass slide). Use a small screw driver (included in the laser kit) to adjust the X and Y position of the laser to match the LED light visible through the microscope eyepiece and calibrate the laser. Other lasers, typically used for *in vitro* fertilization, can be substituted for XYClone laser to perforate fertilized eggs.

3.2. Place a KSOM drop plate containing mouse fertilized eggs on the microscope stage. Do not keep the plate outside of the incubator for longer than 15 minutes.

3.3. Look through the microscope eyepiece and ensure that the embryo's zona pellucida is in focus and the laser LED light is visible.

3.4. Move the microscope stage to target the zona with the LED light/laser.

3.5. Using the computer software set XYClone laser to 250 □S.

3.6. Adjust the LED light size to desired dimensions (setting 5 in this experiment).

3.7. Perforate the zona of each fertilized egg thrice with the laser. The zona can be either pierced or thinned. Using the above settings, the laser will produce a hole with a diameter of 10 □m (Figure 2).

Note: Aiming close to the polar body keeps laser away from the embryonic cell.

3.8. Allow fertilized eggs to recover for 2 hours in the tissue culture incubator before moving to the next step.

4. Transduction of Mouse Fertilized Eggs following XYClone Laser Perforation

4.1. Pipet 2 µl of concentrated lentivirus (greater than 1e8 TU/ml titer) into the 50 µl KSOM drop. Do not pipet up and down. Fertilized eggs readily attach to the pipet tip. Based on our experience, 1e5-5e5 transducing units of lentivirus in a volume less than 3 µl is optimal for gene delivery.

4.2. Allow fertilized eggs to develop into blastocyst for 4 days in the incubator. No need to change the media.

5. Non-Surgical Transfer of Transduced Mouse Embryos to Pseudo-Pregnant Mice

5.1. Use pseudopregnant mice, 3.5 day after mating, prepared in step 1.5.

5.2. Use Non-Surgical Embryo Transfer (NSET) device to implant mouse embryos into pseudopregnant mice. Using a surgical or dissecting microscope, a vaginal speculum is inserted to visualize the cervix. The Non-Surgical Embryo Transfer (NSET) device is inserted approximately 5 mm into the cervix and embryos are deposited in a volume of approximately 2 µL. A sterile NSET device is used for each transfer and discarded after the procedure. All reagents used in the manipulation of the embryos must be sterile.

5.3. Transfer 10-15 healthy blastocyst in 2 µl volume of KSOM to each pseudopregnant mice.

5.4. Continue to monitor and measure the weight gain in pseudopregnant mice in following days to determine whether the NSET was successful.

5.5. Recover pups by allowing the pregnant mice to give birth naturally or performing a caesarean section 17 days after the embryo transfer[25]. A C-section is often necessary if very few embryos are present and they grow too large for natural birth.

5.6. Collect tissue from pups for genotyping to determine the rate of transgenesis [26].

REPRESENTATIVE RESULTS:

Development of isolated/transduced mouse fertilized eggs can be checked under the microscope daily **(Figure 1)**. Healthy embryos develop into blastocyst within 3-4 days. We observed that 60-70% of untreated embryos develop into blastocyst[23]. Out of 114 laser-perforated transduced embryos, 54 developed into blastocyst (rate of 47%) and 46 blastocysts expressed GFP (46/54=85%)[23].

The mouse embryos were laser-perforated on the day of harvest. The optimal setting for the laser treatment was 3 holes per fertilized egg and 250 □s. The laser should be aimed to thin the zona instead of creating a hole **(Figure 2)**. This allows the developing embryos to benefit from an intact encapsulating zona pellucida while becoming permissive to lentiviral transduction. In our experiments, we transduced mouse embryos with a recombinant lentivirus that expressed copepod GFP (abbreviated GFP) from an elongation factor 1a promoter. For transduction, 2 ul of lentivirus was introduced into the culture media. Increasing the amount of virus, directly affected the number of transduced embryos that expressed GFP while adversely affecting embryo development into blastocyst[23]. Viral volumes larger than 10% of KSOM drop volume would also adversely affect blastocyst formation (e.g. greater than 5 µl of virus in a 50 µl KSOM drop).

To validate the viability, transduced mouse embryos were non-surgically transferred to pseudopregnant mice. Six separate NSET events, transferring a total of 58 blastocyst, resulted in 9 GFP transgenic pups out of total of 12, yielding 75% rate of transgenesis [23]. The NSET protocol is reported to yield 30-35% live births[27] compared to 21% yield that we observed in our

experiments (12/58). We hypothesize that the lentiviral treatment may have played a role in embryo development and contributed to the low embryo transfer rate. Pups with multiple lentiviral integrations and high expression of GFP were visually green under a blue LED lamp (465-470 nm) **(Figure 3)**. The number of incorporated GFP copies ranged from 0-6 copies and was determined by performing qualitative PCR on isolated chromosomal DNA from pup tissue{Martin, 2018 #45}.

FIGURE LEGENDS:

Figure 1. Development of Transduced Mouse Fertilized Eggs in Culture. The C57BL/6J mouse fertilized eggs were monitored on Day 0 (harvested fertilized egg), Day 1 (two-cell), Day 2 (8-16 cells), Day 3 (morula), and Day 4 (blastocyst). Embryos were transduced with a lentivirus carrying a GFP gene. Presence of Fluorescence in transduced embryos became evident by Day 2-3.

Figure 2. Laser-Treatment of Mouse Fertilized Eggs. Examples of perforating the zona to produce a hole vs thinning of the zona.

Figure 3. Transgenic Mice Expressing GFP. Dark Reader (DR Spot Lamp, DRSL-9S) was used to visualize GFP positive pups in a dark environment. Non-transgenic control pups were added to the group for contrast. Resulting pups (red arrows) were genotyped and they contained from 0-6 copies of the lentiviral gene/GFP.

DISCUSSION:

The ability of the lentiviruses to integrate into their host genome makes them an ideal vector for stable gene delivery. Lentiviral vectors can carry up to 8.5 kilobase pair (kbp) of genetic material that can accommodate cell-specific or inducible promoters, selection markers, or fluorescent moieties. Incorporated genomic material can replicate as part of their host genome and be regulated to express or deactivate at desired time points. These vectors allow for spatiotemporal control over gene expression at various stages of development and brand lentiviruses as powerful tools for gene delivery.

Laser-assisted lentiviral transgenesis is an effective and easy-to-use method for gene expression *in vivo*. This method can be used for *in vivo* protein production, expression of genetically encoded indicators, or functional studies. Compared to other methods, laser-assisted lentiviral gene delivery is as effective as pronuclear microinjection but requires no technical skills or costly microinjection workstations. The XYClone laser is small, portable, and can easily be shared among several laboratories.

Laser-assisted lentiviral gene delivery is stable and not transient, as in electroporation or photoporation of fertilized eggs. Transient gene delivery is more advantages for delivery of CRISPR-Cas9 components or recombinases since extended expression could lead to aberrant consequences. In this protocol, the integrase deficient lentiviruses (IDLVs) can be substituted for transient transduction of mouse fertilized eggs. IDLVs retain lentiviral infectivity but only retain a fraction (less than 8%) of integrative capability of lentiviruses [25]. Laser-assisted lentiviral

transgenesis is an additional gene delivery option for users to evaluate based on their desired outcome.

The major disadvantage of lentiviral transduction is the random insertion of the delivered gene. Also, cells within the same embryo could host multiple lentiviral integrations that leads to mosaicism in the transgenic. Genotyping of the progeny and multiple rounds of planned breeding is necessary to establish a single locus transgenic animal. Similar breeding strategies are also employed in conventional transgenesis methods [26].

A critical step in this protocol is the length of time spent on laser perforation. Mouse fertilized eggs cultured in KSOM cannot be kept outside of the incubator for longer than 15 minutes. A novice user should move the fertilized eggs to M2 medium, use Advanced KSOM Embryo Medium, or limit the number of embryos per plate. According to our result, 47% of transduced cultured mouse fertilized eggs develop into blastocysts[21]. Therefore, culturing 30-40 fertilized eggs per plate will ensure adequate number of transduced blastocysts in one plate for transfer into pseudopregnant mice.

Laser-assisted perforation of the mouse fertilized egg zona may also be applicable to the zona of other species and allow entry for other types of viruses or transfection reagents.

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DISCLOSURES:

The authors have nothing to disclose.

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Mouse fertilized eggs and early stage embryos are protected by the zona pellucida, a glycoprotein matrix that forms a barrier against gene delivery. This article~~Here, we~~ describes a protocol for perforating the zona with a laser to transduce embryonic cells with lentiviral vectors and to create transgenic mice.

LONG ABSTRACT:

Lentiviruses are efficient vectors for gene delivery to mammalian cells. Following transduction, the lentiviral genome is stably incorporated into the host chromosome and is passed on to progeny. Thus, they are ideal vectors for creation of stable cell lines, *in vivo* delivery of indicators, and transduction of single cell fertilized eggs to create transgenic animals. However, mouse fertilized eggs and early stage embryos are protected by the zona pellucida, a glycoprotein matrix that forms a barrier against lentiviral gene delivery. Lentiviruses are too large to penetrate the zona and are typically delivered by microinjection of viral particles into the perivitelline cavity, the space between the zona and the embryonic cells. The requirement for highly skilled technologists and specialized equipment has minimized the use of lentiviruses for gene delivery to mouse embryos. This article~~Here, we describe a~~ describes a protocol for permeabilizing the mouse fertilized eggs by perforating the zona with a laser. Laser-perforation does not result in any damage to embryos and allows lentiviruses to gain access to embryonic cells for gene delivery. Transduced embryos can develop into blastocyst *in vitro,* and if implanted in pseudopregnant mice, develop into transgenic pups. The laser used in this protocol is effective and easy to use. Genes delivered by lentiviruses stably incorporate into mouse embryonic cells and are germline transmittable. This is an alternative method for creation of transgenic mice that requires no micromanipulation and microinjection of fertilized eggs.

INTRODUCTION:

~~Here, we describe a~~This method provides detailed instructions for permeabilizing the zona pellucida of mouse fertilized eggs to make embryonic cells accessible for gene delivery by lentiviruses. Lentiviruses are designed by nature for efficient gene delivery to mammalian cells. They infect dividing and non-dividing cells and integrate the lentiviral genome into their host chromosomes[1]. The range of lentiviral host cells is readily expanded by pseudotyping the recombinant lentivirus with the vesicular stomatitis virus glycoprotein (VSV-G), due to the broad tropism of the VSV-G protein[2]. Following transduction, lentiviral genes are stably integrated and expressed as part of their host chromosomes creating an ideal tool for generating transgenic animals. If delivered to early stage embryonic cells, the lentiviral genome is replicated and expressed in the entire organism. Lentiviral transduction has led to the production of mice, rat, chicken, quail and pig[3-7] among other species of transgenics. The typical method of lentiviral gene delivery, however, requires skilled technicians and specialized equipment to overcome the zona pellucida barrier that encapsulates the early stage embryos. The overall goal of this method is to describe how to permeabilize the zona using a laser to facilitate lentiviral gene delivery.

Mammalian eggs are surrounded by the zona pellucida which hardens following fertilization to protect the fertilized eggs against polyspermy and to limit environmental interactions[8,9]. The zona forms a barrier that keeps lentiviruses away from the embryonic cells until the embryos are hatched as a blastocyst. Cultured mouse fertilized eggs hatch after 4 days and must be implanted into pseudopregnant mice prior to hatching for normal development into pups. Therefore, for transduction, lentiviruses are microinjected before hatching from the zona into the perivitelline cavity, the space between the zona and the embryonic cells.

The zona pellucida is often removed for *in vitro* fertilization of human eggs to increase the fertilization rate[10]. However, chemical removal of mouse zona pellucida adversely affects mouse embryo development and is harmful to embryonic cells[11,12]. Other methods for gene delivery to mouse fertilized eggs overcome the zona pellucida barrier by direct microinjection of DNA into the cell nucleus [13]. Pronuclear microinjection is an efficient means of delivering genes to embryos. However, since each embryo is held in place individually for microinjection, the practice can be laborious and time consuming for a novice user.

Other methods such as electroporation and photoporation are useful for transient and short-term gene delivery to mouse fertilized eggs[14-16]. These methods are extensively used for delivering CRISPR-Cas9 components and recombinases. However, electroporation and photoporation delivery of genes cannot be used efficiently to create transgenics. Spermatozoa that are collected from punctured mouse epididymis can also be transduced by lentiviruses and used for *in vitro* fertilization to produce transgenic animals[17-20].

~~Here, we facilitated~~In this protocol, the lentiviral gene delivery to mouse embryos is facilitated by permeabilizing the zona using a laser. The XYClone laser was developed as an aid for *in vitro* fertilization[21] and cultivation of embryonic stem cells[22]. It is a small apparatus that is simple to setup and easy to use. Once installed on a microscope, it occupies the space of an objective lens and the accompanying software allows for aiming the laser while looking through the microscope eyepieces (see PROTOCOL: section 3). Once the zona is perforated by the XYClone laser, lentiviruses can be introduced into the culture media for gene delivery[23]. Multiple lentiviruses could be used to simultaneously deliver several genes for chromosomal incorporation.

This protocol will describe how to isolate and culture mouse fertilized eggs, illustrates the use of laser for perforation of the zona pellucida, and demonstrates the transduction of mouse embryonic cells by lentiviruses.

PROTOCOL:

All animal procedures and treatments used in this protocol were in compliance with the NIH/NIEHS animal care guidelines and were approved by the Animal Care and Use Committee (ACUC) at the NIH/NIEHS, Animal Protocol 2010-0004.

1. Preparations

1.1. Prepare/purchase recombinant non-propagating lentiviruses carrying your gene of interest. In this study, lentivirus SBI511 expressing copepod green fluorescent protein (copGFP; abbreviated to GFP) from an elongation factor 1a promoter was used for transduction. Standard protocols[2] were used to produce and titer lentiviruses at titers higher than 1e8 transducing units per m~~l~~L (TU/mL~~l~~).

Note: Use caution and bleach all material that have been in contact with lentiviruses. Refer to your institute's guidelines for safe use and handling of lentiviruses.

1.2. Setup breeding pairs of desired mouse strain the day before harvesting embryos. In this experiment, C57BL/6J strain of mice was used. Female mice used for ovaries and oviduct collection were not treated with any hormones for superovulation.

1.3. 2-24 h~~ours~~ prior to harvesting mouse fertilized eggs, prepare several Potassium Simplex Optimized Medium[24] (KSOM) drop plates as follows: add a 50 µL~~l~~ drop of KSOM medium to the middle of a 35 mm tissue culture-treated dish and cover with 2 mL~~l~~ of Dimethylpolysiloxane (DMPS5X) and place at 37 ^{o}C, 5% CO_2, 5% O_2 and 90% N_2 to equilibrate.

Note: Use disposable sterile plates and discard following exposure to lentiviruses.

1.4. Prepare 1x solution of hyaluronidase in M2 medium from stock solution (100x, 30 mg/mL~~l~~, store at -20 °C). 100x stock solution was prepared in water.

1.5. 24 ~~hours~~h post laser-assisted transduction of mouse fertilized eggs, set up breeding pairs between vasectomized male and female mice to prepare pseudopregnant female mice.

2. Isolation of Mouse Fertilized Eggs

2.1. 16-24 h~~ours~~ post mating, select female mice with vaginal plugs indicative of successful mating~~,~~ and humanely euthanize~~, and isolate ovaries and oviducts according to standard protocols~~[25]. The mice used in this study were euthanized by cervical dislocation under deep CO_2 or isoflurane inhalation.

2.2. Isolate ovaries and oviducts according to standard protocols[25].

2.3~~2~~. Transfer the ovaries and oviducts to a 35 mm dish containing M2 media.

2.4~~3~~. For each ovary, tear ampulla apart to release fertilized eggs surrounded by cumulus cells.

2.5~~4~~. Transfer fertilized eggs and cumulus cells to a 35 mm dish containing 1x hyaluronidase in M2 medium and incubate at room temperature for 3-5 min~~utes~~.

2.6~~5~~. Pipet fertilized eggs up and down to release the cumulus cells.

2.7~~6~~. Transfer fertilized eggs to a 35 mm dish containing M2 media and pipet up and down to wash off hyaluronidase.

2.8~~7~~. Transfer fertilized eggs to a 35 mm dish containing KSOM and pipet up and down to wash off the remaining M2 media and hyaluronidase.

2.9~~8~~. Transfer fertilized eggs to the prepared KSOM drop plates from step 1.3.

Note: Mouse fertilized eggs in KSOM must be kept in a tissue culture incubator at 37 °C, 5% CO_2, 5% O_2 and 90% N_2 to equilibrate. Removing plates from the incubator for longer than 15 min~~utes~~ will result in damage to embryos.

2.9. Allow fertilized eggs to recover for 2 h~~ours~~ in the incubator before moving to the next step.

3. Perforation of Mouse Fertilized Eggs with XYClone Laser

3.1. Setup and calibrate XYClone laser according to the manufacturer's recommendation. ~~Briefly,~~ Other lasers, typically used for *in vitro* fertilization, can be substituted for XYClone laser to perforate fertilized eggs.

~~3.1.1. a~~Attach the laser controller box wire to the laser apparatus on the microscope.

3.1.2. Attach the laser controller box to the computer running the laser software via a USB port.

3.1.3. Plug in the laser controller and switch it on.

3.1.4. Looking through the eyepiece, perforate a test sample (e.g. dry-erase markings on a glass slide).

3.1.5. Use a small screw driver (included in the laser kit) to adjust the X and Y position of the laser to match the LED light visible through the microscope eyepiece and calibrate the laser. ~~Other lasers, typically used for *in vitro* fertilization, can be substituted for XYClone laser to perforate fertilized eggs.~~

3.2. Place a KSOM drop plate containing mouse fertilized eggs on the microscope stage. Do not keep the plate outside of the incubator for longer than 15 min~~utes~~.

3.3. Look through the microscope eyepiece and ensure that the embryo's zona pellucida is in focus and the laser LED light is visible.

3.4. Move the microscope stage to target the zona with the LED light/laser.

3.5. Using the computer software set XYClone laser to 250 ~~²s~~µs.

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3.6. Adjust the LED light size to desired dimensions (setting 5 in this experiment).

3.7. Perforate the zona of each fertilized egg thrice with the laser. The zona can be either pierced or thinned. Using the above settings, the laser will produce a hole with a diameter of 10 µ~~²~~m (Figure 2).

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Note: Aiming close to the polar body keeps laser away from the embryonic cell.

3.8. Allow fertilized eggs to recover for 2 h~~ours~~ in the tissue culture incubator before moving to the next step.

4. Transduction of Mouse Fertilized Eggs following XYClone Laser Perforation

4.1. Pipet 2 µl~~l~~L of concentrated lentivirus (greater than 1e8 TU/m~~l~~L titer) into the 50 µ~~l~~L KSOM drop. Do not pipet up and down. Fertilized eggs readily attach to the pipet tip. Based on our experience, 1e5-5e5 transducing units of lentivirus in a volume less than 3 µ~~l~~L is optimal for gene delivery.

4.2. Allow fertilized eggs to develop into blastocyst for 4 days in the incubator. No need to change the media.

5. Non-Surgical Transfer of Transduced Mouse Embryos to Pseudo-Pregnant Mice

5.1. Use pseudopregnant mice, 3.5 day after mating, prepared in step 1.5.

5.2. Use Non-Surgical Embryo Transfer (NSET) device to implant mouse embryos into pseudopregnant mice.

5.2.1. Using a surgical or dissecting microscope, insert a vaginal speculum ~~is inserted~~ to visualize the cervix.

5.2.2. Insert ~~T~~the Non-Surgical Embryo Transfer (NSET) device ~~is inserted~~ approximately 5 mm into the cervix and deposit embryos ~~are deposited~~ in a volume of approximately 2 µL.

Note: A sterile NSET device is used for each transfer and discarded after the procedure. All reagents used in the manipulation of the embryos must be sterile.

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5.3. Transfer 10-15 healthy blastocyst in 2 µ~~l~~L volume of KSOM to each pseudopregnant mice.

5.4. Continue to monitor and measure the weight gain in pseudopregnant mice in following days to determine whether the NSET was successful.

5.5. Recover pups by allowing the pregnant mice to give birth naturally or performing a caesarean section 17 days after the embryo transfer[26]. A C-section is often necessary if very few embryos are present and they grow too large for natural birth.

5.6. Collect tissue from pups for genotyping to determine the rate of transgenesis [27].

REPRESENTATIVE RESULTS:

Development of isolated/transduced mouse fertilized eggs can be checked under the microscope daily **(Figure 1)**. Healthy embryos develop into blastocyst within 3-4 days. In this protocol, ~~We observed that~~ 60-70% of untreated embryos develop into blastocyst[23]. Out of 114 laser-perforated transduced embryos, 54 developed into blastocyst (rate of 47%) and 46 blastocysts expressed GFP (46/54=85%)[23].

The mouse embryos were laser-perforated on the day of harvest. The optimal setting for the laser treatment was 3 holes per fertilized egg and 250 µ~~l~~s. The laser should be aimed to thin the zona instead of creating a hole **(Figure 2)**. This allows the developing embryos to benefit from an intact encapsulating zona pellucida while becoming permissive to lentiviral transduction. In

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these~~our~~ experiments, ~~we transduced~~ mouse embryos were transduced with a recombinant lentivirus that expressed copepod GFP (abbreviated GFP) from an elongation factor 1a promoter. For transduction, 2 u~~l~~L of lentivirus was introduced into the culture media. Increasing the amount of virus, directly affected the number of transduced embryos that expressed GFP while adversely affecting embryo development into blastocyst[23]. Viral volumes larger than 10% of KSOM drop volume would also adversely affect blastocyst formation (e.g. greater than 5 µ~~l~~L of virus in a 50 µ~~l~~L KSOM drop).

To validate the viability, transduced mouse embryos were non-surgically transferred to pseudopregnant mice. Six separate NSET events, transferring a total of 58 blastocyst, resulted in 9 GFP transgenic pups out of total of 12, yielding 75% rate of transgenesis [23]. The NSET protocol is reported to yield 30-35% live births[28] compared to 21% yield that were observed in our experiments (12/58). ~~We hypothesize that t~~The lentiviral treatment may have played a role in embryo development and contributed to the low embryo transfer rate. Pups with multiple lentiviral integrations and high expression of GFP were visually green under a blue LED lamp (465-470 nm) **(Figure 3)**. The number of incorporated GFP copies ranged from 0-6 copies and was determined by performing qualitative PCR on isolated chromosomal DNA from pup tissue[23].

FIGURE LEGENDS:

Figure 1. Development of Transduced Mouse Fertilized Eggs in Culture. The C57BL/6J mouse fertilized eggs were monitored on Day 0 (harvested fertilized egg), Day 1 (two-cell), Day 2 (8-16 cells), Day 3 (morula), and Day 4 (blastocyst). Embryos were transduced with a lentivirus carrying a GFP gene. Presence of Fluorescence in transduced embryos became evident by Day 2-3.

Figure 2. Laser-Treatment of Mouse Fertilized Eggs. Examples of perforating the zona to produce a hole vs thinning of the zona.

Figure 3. Transgenic Mice Expressing GFP. Dark Reader (DR Spot Lamp, DRSL-9S) was used to visualize GFP positive pups in a dark environment. Non-transgenic control pups were added to the group for contrast. Resulting pups (red arrows) were genotyped and ~~they~~ contained from 0-6 copies of the lentiviral gene/GFP.

DISCUSSION:

The ability of the lentiviruses to integrate into their host genome makes them an ideal vector for stable gene delivery. Lentiviral vectors can carry up to 8.5 kilobase pair (kbp) of genetic material that can accommodate cell-specific or inducible promoters, selection markers, or fluorescent moieties. Incorporated genomic material can replicate as part of their host genome and be regulated to express or deactivate at desired time points. These vectors allow for spatiotemporal control over gene expression at various stages of development and brand lentiviruses as powerful tools for gene delivery.

Laser-assisted lentiviral transgenesis is an effective and easy-to-use method for gene expression *in vivo*. This method can be used for *in vivo* protein production, expression of genetically encoded indicators, or functional studies. Compared to other methods, laser-assisted lentiviral gene

delivery is as effective as pronuclear microinjection but requires no technical skills or costly microinjection workstations. The XYClone laser is small, portable, and can easily be shared among several laboratories.

Laser-assisted lentiviral gene delivery is stable and not transient, as in electroporation or photoporation of fertilized eggs. Transient gene delivery is more advantages for delivery of CRISPR-Cas9 components or recombinases since extended expression could lead to aberrant consequences. In this protocol, the integrase deficient lentiviruses (IDLVs) can be substituted for transient transduction of mouse fertilized eggs. IDLVs retain lentiviral infectivity but only retain a fraction (less than 8%) of integrative capability of lentiviruses [29]. Laser-assisted lentiviral transgenesis is an additional gene delivery option for users to evaluate based on their desired outcome.

The major disadvantage of lentiviral transduction is the random insertion of the delivered gene. Also, cells within the same embryo could host multiple lentiviral integrations that leads to mosaicism in the transgenic. Genotyping of the progeny and multiple rounds of planned breeding is necessary to establish a single locus transgenic animal. Similar breeding strategies are also employed in conventional transgenesis methods [30].

A critical step in this protocol is the length of time spent on laser perforation. Mouse fertilized eggs cultured in KSOM cannot be kept outside of the incubator for longer than 15 min~~utes~~. A novice user should move the fertilized eggs to M2 medium, use Advanced KSOM Embryo Medium, or limit the number of embryos per plate. According to our result, 47% of transduced cultured mouse fertilized eggs develop into blastocysts[23]. Therefore, culturing 30-40 fertilized eggs per plate will ensure adequate number of transduced blastocysts in one plate for transfer into pseudopregnant mice.

Laser-assisted perforation of the mouse fertilized egg zona may also be applicable to the zona of other species and allow entry for other types of viruses or transfection reagents.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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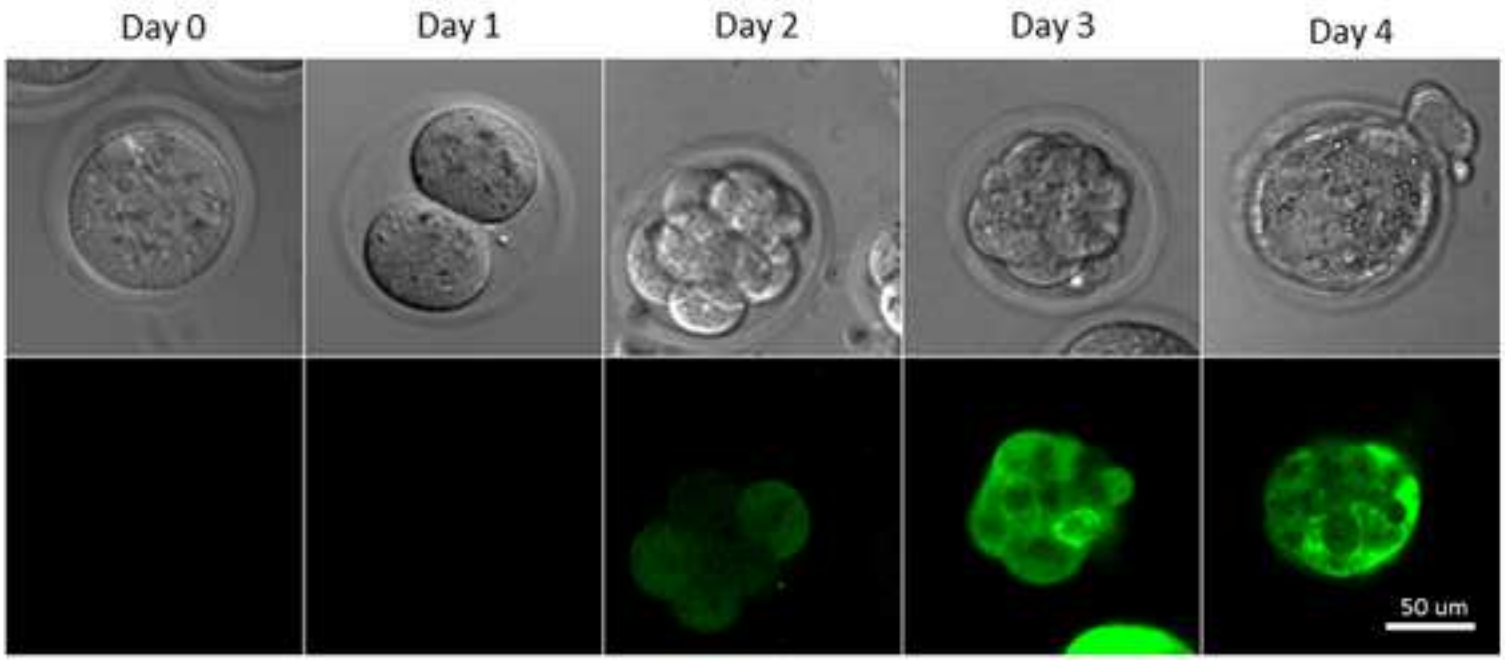

Figure 2

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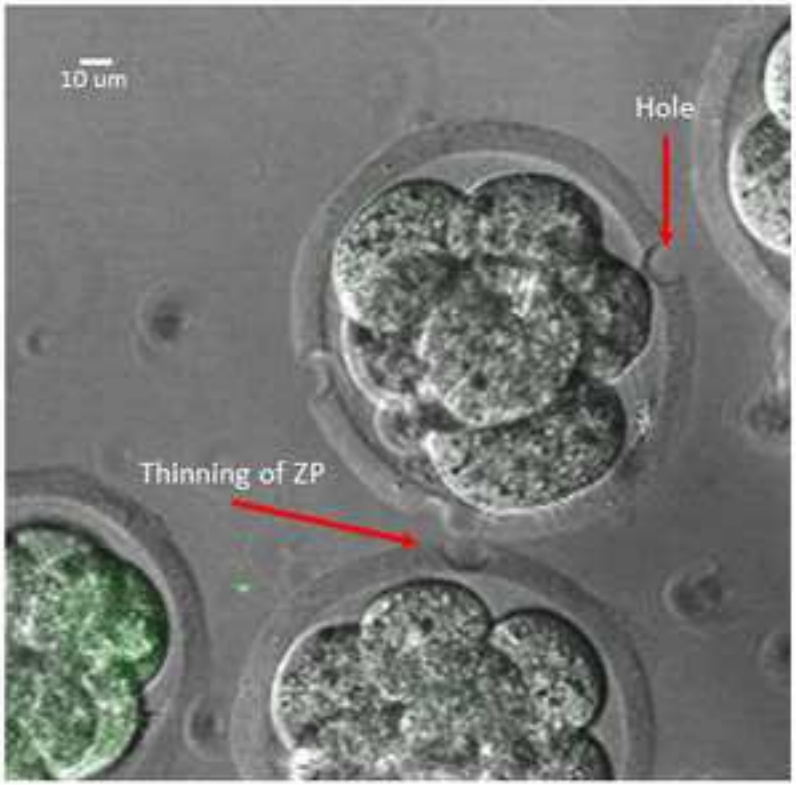

Figure 3

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Figure 1 - revision 2

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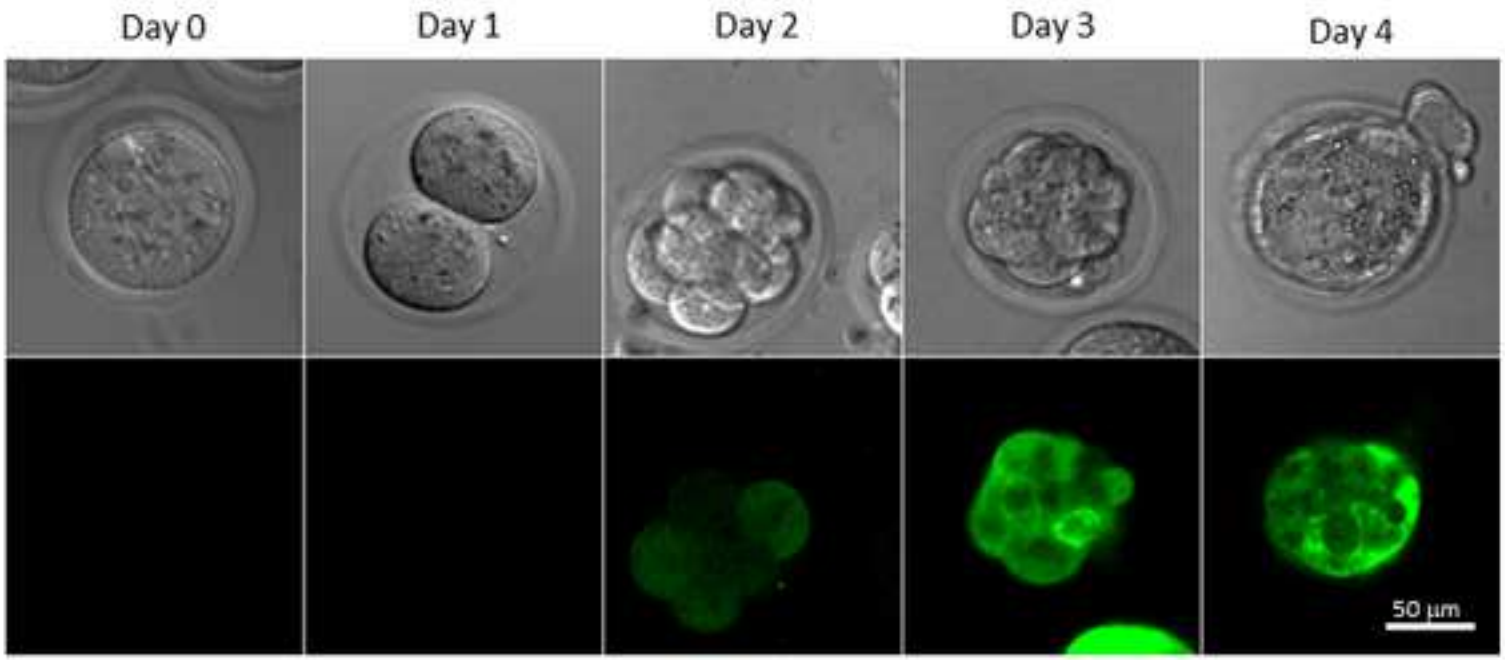

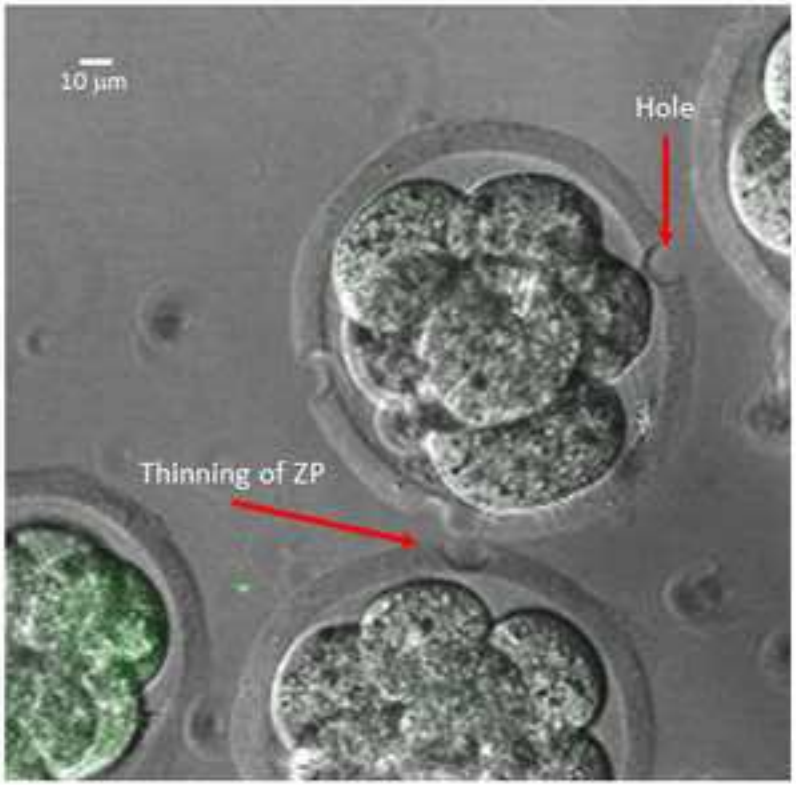

Figure 3 - revision 2

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Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
CD510B-1 plasmid	System Biosciences	CD510B-1
KSOM medium	Millipore	MR-020P-5F
M2 medium	Millipore	MR-015-D
Dimethylpolysiloxane	Sigma	DMPS5X
hyaluronidase	Sigma	H3506
XYClone Laser	Hamilton Thorne Biosciences	
Non-Surgical Embryo Transfer (NSET) Device	ParaTechs	60010

Comments/Description
used to package the lentivirus expressing EF1a-copGFP
culturing embryos
culturing embryos
culturing embryos
used to remove cumulus cells
perforating mouse fertilized eggs
NSET of embryos

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Dimethylpolysiloxane	Sigma	DMPS5X
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XYClone Laser	Hamilton Thorne Biosciences	
Non-Surgical Embryo Transfer (NSET) Device	ParaTechs	60010
KSOM medium	Millipore	MR-020P-5F
M2 medium	Millipore	MR-015-D

Comments/Description

used to package the lentivirus expressing EF1a-copGFP
culturing embryos
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culturing embryos

Composition of KSOM:

Reagent Name	mg/100mL
NaCl	555
KCl	18.5
KH2PO4	4.75
MgSO4 7H2O	4.95
CaCl2 2H2O	25
NaHCO3	210
Glucose	3.6
Na-Pyruvate	2.2
DL-Lactic Acid, sodium salt	0.174mL
10mM EDTA	100µL
Streptomycin	5
Penicillin	6.3
0.5% phenol red	0.1mL
L-Glutamine	14.6
MEM Essential Amino Acids	1mL
MEM Non-essential AA	0.5mL
BSA	100

culturing embryos

Composition of M2:

Reagent Name	mg/100mL
Calcium Chloride	25.1
Magnesium Sulfate (anhydrous)	16.5
Potassium Chloride	35.6

Potassium Phosphate, Monobasic	16.2
Sodium Bicarbonate	35
Sodium Chloride	553.2
Albumin, Bovine Fraction	400
D-Glucose	100
Na-HEPES	54.3
Phenol Red	1.1
Pyruvic Acid	3.6
DL-Lactic Acid	295

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Article Title:	Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs
Signature:	Negin P. Martin -S Digitally signed by Negin P. Martin -S Date: 2018.04.13 09:32:55 -04'00'
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To: *Journal of Visualized Experiments (JoVE)*

From: Negin P. Martin, Ph.D.

Subject: Manuscript JoVE58327 - Editorial and Peer Review Rebuttal

June 21th, 2018

Dear Editor Dr. Wu and Reviewers,

Many thanks for taking the time to review our manuscript. Please find our revised manuscript entitled " Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs" attached. We hope that the new revisions address all comments and concerns. We have reviewers' comments and our improvements to the paper listed below.

I appreciate your time and should you have any questions or concerns please feel free to contact me.

Sincerely yours,
Negin P. Martin, Ph.D.

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Done.
2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. We have 3 figures uploaded as .tiff files.
3. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient. Done.
4. Figure 1: Please label in Figure 1 the days when the images were taken. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. The figures were marked with Day 0-4 and a scale bar was added to the image.
5. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available. Done – no antibodies used.
6. Please provide an email address for each author. Done – on the first page of manuscript.
7. Introduction: Please rephrase the Introduction to include a clear statement of the overall goal of this method. The introduction was modified to the best of our abilities and we hope that the purpose is clearly stated in our new version. Please feel free to contact me if you need further modifications.
8. Please define all abbreviations before use. Done.
9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, °C, etc. Done.
10. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc. Done.
11. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., H2O, CO2, etc. Done.

12. Please place the ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee. Done.
13. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. All company names and catalog numbers were removed.
For example: Hamilton Thorne Biosciences, System Biosciences, Charles River, Millipore, Sigma, Molecular Biotechnology, etc.
14. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please see manuscript for the added protocol details.
15. 2.1: Please specify the euthanasia method and describe how to isolate ovaries. Done.
16. 2.4: What is the incubation temperature? Done.
17. 5.2: Please remove the weblink which contains commercial information. Done.
18. 5.5: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action. More details and references were added.
19. References: Please do not abbreviate journal titles. We used Endnote JoVE style in the latest version. But, it still abbreviates the journal titles. Is there another style that we should use?

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Reviewer #1:

Manuscript Summary:
In this manuscript, the authors described a novel method to generate transgenic mice using lentiviruses. They used laser to punch a hole on zona pellucida, and then cultured these punched embryos with lentiviruses. Based on their result, the authors could obtain ~70% transgene rate, and declared this method would be more friendly for researchers without embryo micromanipulation.

Major Concerns:
None

Minor Concerns:
1. In line 128, the authors should make sure whether they prepare 1x solution of hyaluronidase "in water". Great point. We prepared 100x stock in water and then dilute to 1x in M2. This was added to the protocol steps.
2. In line 138, the authors should take ovaries and oviducts. Corrected in the manuscript to reflect Ovaries and Oviducts.
3. In line 177, the authors should describe the size of "holes" the laser punctured, since this is the key point of this technique. The size of the hole, diameter 10 um, was added to the manuscript. Also, Figure 2 was modified to show an actual picture of the holes with scale bar.
4. In line 187, the authors should describe the volume of KSOM drop since it is highly related to the concentration of lentiviruses. The authors should also describe the optimal concentration they suggest here. All requested information was added.
5. In line 230, the authors may not need to describe "Number of integrated lentiviral genome copies was validated by genotyping the pups tail samples" since they did not should this result. We removed the statement and also included the result of our genotyping for readers.
6. In line 229, the embryo transfer rate is relatively low (12/58). The PI may provide opinions for this aspect. We hypothesize that the lentiviral treatment may have played a role in embryo development and contributed to the low embryo transfer rate. The viral treatment has an adverse effect on blastocyst formation and the transduction may also impede development. We also included references from published literature about the expected rate of embryo transfer with NSET and our results.
7. In line 249, the authors should label in different images and describe the different developmental stages of fertilized oocytes in the figure legend. All requested information was added to Figure 1 and the representative result section in the manuscript.

8. In figure 2, it would be better to provide the real images. This was a great improvement. The new version of Figure 2 has an actual picture of the perforated embryos with a scale bar.
9. In figure 3, the authors need to label these mice in the image. The transgenic pups were labeled with an arrow in Figure 3 and the genotyping result was added to the figure legend.

Reviewer #2:

Manuscript Summary:

The report describes the use of a laser equipment (XYclone Laser System) for delivery of a transgene construction into the mice zygote by perforation of the zona pellucida. The idea is into the scope of the journal. However, this information was reported by the same authors this year in Transgenic Research, showing different technical parameters with different laser treatments performed in different experiments. In addition, graphic material (i.e. photos and diagrams) were also reported in that paper. So, the question is which is the new information about the method or technique reported previously? In my opinion, the contribution of the current MS is not too different than the method available in the previous paper.

After the publication of our paper in *Transgenic Research*, we were approached by *JoVE* to prepare a video demonstrating the procedure for perforating the zona pellucida of the mouse fertilized eggs. We hope to provide more details and demonstrate how the zona is perforated for lentiviral gene delivery in this publication.

As called to attention by the editor, novelty is not a requirement for publication in *JoVE*. To give an example, the video about transformation of bacteria in *JoVE* is regularly watched by our students during first week of laboratory orientation. It is not novel but provides a wealth of information, a detailed protocol, and a visual demonstration of the method. *JoVE* videos are an amazing educational tool for researchers.

Reviewer #3:

Manuscript Summary:

The MS presents an interesting method using laser to thin the zona pellucida of early embryos and perform lentiviral transduction without the need of laborious embryo micromanipulation. The method is already used in several laboratories and species, with good results. The MS is well written, english is clear and the methods as well described. In order to help the authors improve the MS, I wrote below some recommendations:

Major Concerns:

- XY Clone is a commercial name, I recommend you to use a generic name throughout the MS since this protocol should be carried out using any brand of laser. We agree with the reviewer that any laser used for *in vitro* fertilization can be substituted in place of XYClone laser. We replaced all "XYclone laser" with "laser" in manuscript except for material for section 3 of the protocol where we demonstrate the perforation. In section 3, we had to give detailed information about how to setup and perforate the zona. Each laser is setup differently, so we left the XYClone laser in that section but added the statement: "Other lasers, typically used for *in vitro* fertilization, can be substituted for XYClone laser to perforate fertilized eggs." at the end of the protocol step 3.1.

- I would not say either that the laser is economical (abstract, line 49), since several laboratories cannot afford this cost. Our laboratory is very small with limited resources. Since the laser can be used for gene delivery (Viral Vector Core), *in vitro* fertilization (Comparative Medicine Branch), and embryo dissection (Stem Cell Biology), two of our departments combined resources to buy the laser. The laser is very small and similar in size to an objective lens. It is setup within a few minutes and can be transported and shared among several labs easily. In contrast, microinjection stations at our institute are not shared among biologists in different labs. The economical reference is because the laser is half the price of a microinjection station (based on pricing in our institute) and the cost can be shared among several laboratories/departments. However, we respect reviewer's wishes and removed the comment about the laser being economical. Please note that we have no relationship with the XYClone manufacturers.

- Regarding representative results, please include how many embryos were incubated with lentivirus and how many were GFP+ at morula/blastocyst stage (in vitro efficiency). We included the requested result in the representative data section.
- It seems that very few pups were born after embryo transfer, could this be due to some problems in the embryos linked to the use of lentivirus? or rather due to the use of NSET? Please clarify. We hypothesize that the lentiviral

treatment may have played a role in embryo development and contributed to the low embryo transfer rate. The viral treatment has an adverse effect on blastocyst formation and therefore, transduction may also impede development. We also included references from published literature about the expected rate of embryo transfer with NSET and our results.
- Please include the number of integrated lentiviral genome copies in the representative results to evaluate overall efficiency. This data was added to the manuscript.

Minor Concerns:
Introduction
Line 63: include "among other species" after pig, since other transgenic species have been produced also Done
Line 75-76: include other references for this statement, since removal of zona pellucida is a standard procedure in several techniques without affecting embryo development or causing any harm. Our apologies. We only use murine embryos for research at our institute and we meant to discuss mouse fertilized eggs without zona in that statement. Fertilized eggs of larger mammals, for example humans, develop well without the zona. We corrected the text to reflect the above and included a reference for the mouse zona in the manuscript.

Results
Since the used lentiviral vectors had GFP, it would be good to include in vitro efficiency before embryo transfer (how many GFP embryos/total infected embryos) This result was included in the representative data section.

Protocol
1.2 : The mice need to be superovulated first, please include that if necessary. We did not super ovulate the mice in these experiments. This was added to the protocol.
2.1 : change "isolate ovaries" for "isolate ovaries and oviduct" Done
4.1 : lentiviral vectors remain in the KSOM drop until blastocyst development? Please clarify if the media needs to be changed. We added to the protocol that the media is not changed during the entire experiment.

Materials table
Hyaluronidase is duplicated, please delete one Done

DEPARTMENT OF HEALTH AND HUMAN SERVICES

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To: *Journal of Visualized Experiments (JoVE)*

From: Negin P. Martin, Ph.D.

Subject: Manuscript JoVE58327 - Editorial Review Rebuttal

July 9th, 2018

Dear Editor Dr. Wu,

Thank you again for taking the time to review our manuscript. Please find our revised manuscript entitled " Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs" attached. We hope that the new revisions address all comments and concerns. We have your comments and our improvements to the paper listed below.

I appreciate your time and should you have any questions or concerns please feel free to contact me.

Sincerely yours,
Negin P. Martin, Ph.D.

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done.
2. Please do not abbreviate journal titles for all references. Done.
3. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Done. All personal pronouns were removed.
4. 4. Please use h, min, s for time units. Done.
5. 5. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc. Done. The only unit left that is not SI is Transducing Unit/mL. There is no SI unit for lentiviral concentration and TU/mL is commonly used to depict the virus amount.
6. Step 1.3: What's the composition of Potassium Simplex Optimized Medium? Composition of KSOM was added to the Material list.
7. 1.4: What's the composition of M2 medium? Composition of M2 was added to the Material list.
8. 5.2: Please ensure that all text is written in imperative tense. Done.
9. Please split some long steps so that each step contains 2-3 actions and is less than 4 lines. Done.
10. Figure 1: Please use µm instead of um. Done.
11. Figure 2: Please use µm instead of um. Done.

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