Video Article

Laser-assisted Cytoplasmic Microinjection in Livestock Zygotes

Yanina S. Bogliotti[1], Marcela Vilarino[1], Pablo J. Ross[1]

[1]Department of Animal Science, University of California, Davis

Correspondence to: Pablo J. Ross at pross@ucdavis.edu

URL: https://www.jove.com/video/54465
DOI: doi:10.3791/54465

Keywords: Genetics, Issue 116, Cytoplasmic microinjection, laser, zygote, bovine, ovine, gene disruption, genome editing

Date Published: 10/5/2016

Citation: Bogliotti, Y.S., Vilarino, M., Ross, P.J. Laser-assisted Cytoplasmic Microinjection in Livestock Zygotes. *J. Vis. Exp.* (116), e54465, doi:10.3791/54465 (2016).

Abstract

Cytoplasmic microinjection into one-cell embryos is a very powerful technique. As an example, it enables the delivery of genome editing tools that can create genetic modifications that will be present in every cell of an adult organism. It can also be used to deliver siRNA, mRNAs or blocking antibodies to study gene function in preimplantation embryos. The conventional technique for microinjecting embryos used in rodents consists of a very thin micropipette that directly penetrates the plasma membrane when advanced into the embryo. When this technique is applied to livestock animals it usually results in low efficiency. This is mainly because in contrast to mice and rats, bovine, ovine, and porcine zygotes have a very dark cytoplasm and a highly elastic plasma membrane that makes visualization during injection and penetration of the plasma membrane hard to achieve. In this protocol, we describe a suitable microinjection method for the delivery of solutions into the cytoplasm of cattle zygotes that has proved to be successful for sheep and pig embryos as well. First, a laser is used to create a hole in the zona pellucida. Then a blunt-end glass micropipette is introduced through the hole and advanced until the tip of the needle reaches about 3/4 into the embryo. Then, the plasma membrane is broken by aspiration of cytoplasmic content inside the needle. Finally, the aspirated cytoplasmic content followed by the solution of interest is injected back into the embryonic cytoplasm. This protocol has been successfully used for the delivery of different solutions into bovine and ovine zygotes with 100% efficiency, minimal lysis, and normal blastocysts development rates.

Video Link

The video component of this article can be found at https://www.jove.com/video/54465/

Introduction

Cytoplasmic microinjection of 1-cell embryos is a very powerful technique. It can be used for delivering any solution into the embryo to, for example, produce gene knock-outs to study gene function or to generate gene-edited animals. Most agriculturally-relevant farm animal zygotes have a very high fatty acid composition that makes their cytoplasm opaque and dark[1]. They also have a fairly elastic plasma membrane (PM). These characteristics make microinjection using conventional pronuclear/cytoplasmic injection as used in rodent species challenging and often inaccurate.

Cytoplasmic microinjection has advantages over pronuclear microinjection since it is easier to perform and also causes less damage to the injected embryos, resulting in higher viability[2]. The overall goal of this protocol is to demonstrate a successful method for delivering solutions into the cytoplasm of farm animal zygotes. To be able to perform cytoplasmic microinjection with high efficiency on livestock embryos, a laser is used to generate a hole in the zona pellucida (ZP) and then a blunt-end glass needle is used for the microinjection. This strategy aims to reduce the mechanical damage imprinted on the embryo during injection. Then, aspiration of cytoplasmic content inside the injection needle allows efficient and confident breakage of the PM ensuring that the solution is delivered into the cytoplasm of the embryo.

This technique has already been successfully used in bovine embryos to deliver siRNA into the zygotic cytoplasm[3,4] and to generate mutations using the clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated system 9 (Cas9) system[5]. It is also suitable (with minor modifications) to inject bovine cumulus-enclosed oocytes[6]. Here, we describe our injection protocol delivering a dye, that can be applicable to injecting any desired solution into the zygote, and show that using this technique causes minimal lysis and does not affect early embryo development.

Protocol

1. Micropipette Production

1. Injection micropipette
    1. Place a borosilicate glass capillary (outer diameter (OD): 1.0 mm, inner diameter (ID): 0.75 mm) in a micropipette puller (in the center of the right and left capillary holders) and lock it.

2. Use an appropriate program to pull the glass capillary so it results in a thin tip with a long tapper. (Example: Heat: 825; Pull: 30; Velocity: 120; Time: 200; Pressure: 500).
3. Carefully remove the pulled pipettes from the device and place it on a microforge in a horizontal position.
4. Bring the pulled pipette and the microforge heater filament with its glass bead into focus. Use the eyepiece reticle to measure the desired thickness and move the pipette horizontally until the heater filament reaches the target inner diameter (5 µm).
5. Adjust the heat to about 45% and gently bring the pipette down so it touches the filament. Activate the heater briefly. This will slightly melt the pipette so it adheres to the filament and upon cooling, the pipette will break at the contact point generating a straight cut.
   Note: Setting the appropriate temperature is key in this step since temperatures too high will make the pipette bend and thus a straight cut will not be possible and temperatures too low will not be sufficient to melt the pipette (**Figure 1A**).
6. Make an approximate 30° angle near the pipette tip by positioning it about 10 µm away from the heater filament. Set the temperature to 60% and activate the heater.
   Note: This will bend the pipette over the filament. This angle is desired so the tip of the needle is parallel to the surface of the injection plate when mounted into the injector (**Figure 1B**).
7. Handle microinjection pipettes with caution as they are extremely sharp and fragile.

2. Holding micropipette
   1. Place a borosilicate glass capillary (OD: 1.0 mm, ID: 0.75 mm) in a micropipette puller (in the center of the right and left capillary holders) and lock it.
   2. Use an appropriate program to pull the glass capillary so it results in a thin tip with a long even taper and parallel walls (Example: Heat: 815; Pull: 20; Velocity: 140; Time: 175; Pressure: 200).
   3. Carefully remove the pulled pipette from the puller device and place it on the microforge holder in a horizontal position. Adjust the focus on the heater filament and pipette.
   4. Use the eyepiece reticle to measure the pipette diameter and move the pipette until its diameter over the filament reaches 180 µm.
   5. At the indicated size, mark the glass capillary with a diamond tip pen, and then gently press on the pulled tip to break the glass. This should result in a straight cut (**Figure 1C**).
   6. Move the pipette to a vertical position with its tip close to the filament. Set the temperature of the microforge to 60%.
   7. Fire-polish the tip of the pipette using standard techniques[7] until it reaches an ID of 40 µm. Use the eyepiece reticle to check the ID (**Figure 1D**).
   8. Make an angle on the pipette.
      1. Bring the pipette back to a horizontal position about 10 µm away from the filament and 5 mm away from its tip. Set the temperature to approximately 60% and activate the heater to bend the pipette over the filament. Continue heating until a desired angle (of about 30^{o}) is reached. Check the angle visually (**Figure 1E**).
         Note: The pipette is usually mounted to the microinjector at an approximate angle of 60^{o} with respect to the bottom of the injection dish. The tip of the pipette is bent so it is parallel to the bottom of the dish, which is necessary for the correct injection of the embryo at its middle plane.

2. Micromanipulator Setup

1. Check that the microinjectors are fully loaded with oil and that there are no air bubbles in the system (bubbles in the microinjector prevent fine control of injection).
2. Check that the micromanipulators are in the center position. This will allow for a wide range of movement of the pipettes.
3. Insert the holding pipette into the left micromanipulator holder and the injection pipette into the right micromanipulation holder.
4. Allow oil to enter into the pipettes by capillarity and check that the system is working properly by moving oil up and down inside the pipette using the microinjector controls.
   Note: If there is no movement of oil inside the needles, there might be a clog. In this case, use a new needle.
5. Use the micromanipulator controls to bring the pipettes into the center of the microscope's field of view. Using 4X magnification, check that the micropipette tips are in the correct angle (parallel to the bottom of the dish).
   Note: A correct setup of the needles is key for a successful injection and for results consistency.
6. Calibrate the laser system following the manufacturer's calibration manual.

3. Preparation of Injection Dish (Figure 2)

1. Place a 50 µl drop of warmed (37 °C) SOF-HEPES (composition detailed in the materials section) supplemented with 20% fetal bovine serum (FBS) on the center of the lid of a 100 mm petri dish. Place a 1 - 2 µl drop of the solution to be injected close to the injection drop. Ensure that the embryos do not cool down below RT.
   Note: In this protocol we will add the Dextran-Red dye to the injecting solution to be able to visualize the site of injection and track it later.
2. Cover the drops in the injection plate using approximately 10 ml of mineral oil.
3. Place the injection dish on the stage of the inverted microscope and bring the pipettes into the injection drop. Check that the needles are in focus inside the drop. Adjust their height as needed using the micromanipulator controls.
4. Using a microdispenser, load about 20 - 30 zygotes (17 - 20 hr post-fertilization (hpf)) in the upper side of the injection drop. Perform injection at room temperature so the number of embryos in the injection drop is determined by the speed at which the person can inject them. Do not load more embryos than the ones that can be injected in 30 min.
   1. To load the embryos into the microdispenser, depress the plunger fully and immerse the tip into the solution containing the embryos. Touch the embryos to be picked up with the tip of the glass capillary (one at the time) and slowly release the plunger so each embryo enters into the capillary. Repeat this until all the embryos are picked up or the microdispenser capacity is full.

2. To release the loaded embryos, depress the plunger gently until the entire volume containing the embryos is released from the capillary.

4. Microinjection

1. Using the 4X objective, load the solution to be injected into the injection pipette by applying negative pressure (aspirating). Load sufficient solution to inject 2 - 3 zygotes. Then move to the injection drop.
2. Within the injection drop, move the holding pipette so the tip gets close to a zygote. Apply negative pressure (aspirate) with the holding microinjector so the zygote gets fixed to the holding pipette and change to a 20X objective.
3. Once the zygote is attached to the holding pipette, check its quality using the injecting pipette to gently move the embryo around without detaching it. A good quality zygote should have the two polar bodies (although sometimes only one is seen) and a homogeneous cytoplasm. Do not inject abnormal zygotes (**Figure 3A**).
4. If necessary, reposition the embryo for a proper injection location. A good positioning would be that in which there is some space between the ZP and the PM, so the PM does not get damaged by the laser.
5. Check that the injection pipette is positioned on the embryo's mid-plane by gently touching the zygote on the site of injection. If it is not on its mid-plane, the needle will tend to make the embryo rotate instead of puncture. Adjust the height of the injection pipette as needed.
6. Make a hole in the ZP using a laser (**Figure 3B**).
    1. Using the laser's software place the laser reticle in the ZP where the hole will be made (on the opposite side to where the embryo is attached to the holding pipette). Using the laser control window click on the fire button. Ensure that the size of the hole is large enough to create an opening in the ZP for the needle to pass through without damaging the PM (Example: 0.662 msec Pulse width/ 6.9 µm Hole size).
7. Pass the injection needle through the hole in the ZP and make contact with the PM. Continue pushing the pipette forward until the needle is ¾ of the way into the embryo (**Figure 3C**).
8. Observe the position of the meniscus at the solution-oil interphase, as this will be used to consistently control injection volume.
9. Apply negative pressure (aspirate) on the injection pipette, which will result in PM and cytoplasm being aspirated into the injection needle. Continue until the movement of cytoplasm into the needle accelerates or when cytoplasm content mixing up with injection solution can be seen. These will indicate breakage of the PM (**Figure 3D**).
10. Inject back the cytoplasmic content followed by the solution into the cytoplasm of the zygote by applying positive pressure (injecting), until the meniscus at the solution-oil interphase has advanced one zygote's diameter equivalent past the starting point.
    Note: Under our conditions, this represents approximately 7 pl of injected solution (**Figure 3E**).
11. Change to a 4X objective and move the injected embryo/s to the lower side of the injection drop. Release the embryo by applying positive pressure on the holding microinjector. Keep the non-injected embryos on the upper side and the injected ones on the lower side of the drop to help keep track of the injected/non-injected embryos and work efficiently.

5. Embryo Recovery and Injection Results

1. Make 3 drops of approximately 200 µl in a new dish with pre-warmed SOF-HEPES. Collect the injected embryos using a microdispenser (as described above). Wash the injected embryos by moving them through the 3 SOF-HEPES drops.
2. Count the embryos lysed during microinjection and discard them.
    Note: A lysed zygote can be easily distinguished from an intact one since the first one appears translucent, fill out the whole ZP, and it usually has cytoplasmic content leaking to the outside of the embryo.
3. Optionally, check the microinjection efficiency using a fluorescent microscope. Be aware that UV light exposure can induce embryo damage that can affect subsequent development.
4. Culture groups of 25 injected embryos in 50 µl drops of potassium simplex optimization medium (KSOM) supplemented with 4 mg/ml bovine serum albumin (BSA) under mineral oil at 38.5 °C, 5% CO_2 in air, and humidity to saturation.
5. Supplement the culture media with 5% FBS two days after injection.
6. Check blastocyst (BL) rates 7 days after injection. Calculate BL rates as total number of blastocyst embryos/total number of embryos cultured in that drop.

Representative Results

Laser-assisted cytoplasmic microinjection is a powerful and reliable protocol to deliver solutions into the cytoplasm of livestock zygotes. **Figure 3** shows a general outline of the zygotes before and after injection as well as the overall outline of the technique. Dextran-red is used as injecting solution to allow tracking site of injection and injection efficiency and accuracy. Successful delivery of the solution is illustrated in **Figure 4** showing a recently injected embryo in which the dye is homogeneously distributed in the cytoplasm. Using this technique 100% of the embryos are injected in their cytoplasm.

This protocol has demonstrated to have minimal lysis rates in bovine and ovine zygotes. Only 15.6 ± 5 and 5.8 ± 3.9% of the injected embryos were lysed in cattle and sheep, respectively, as a result of microinjection **(Figure 5)**. Also, as shown in **Figure 6**, there are no statistically significant differences in blastocyst development rates in the control versus injected groups for both bovine (32.8 ± 6.6% control, 31.4 ± 5.9% injected) and ovine embryos (40.3 ± 7.8 control, 30.3 ± 6.0% injected).

These results indicate that laser-assisted intracytoplasmic injection causes minimal damage during injection and results in normal blastocyst development rates.

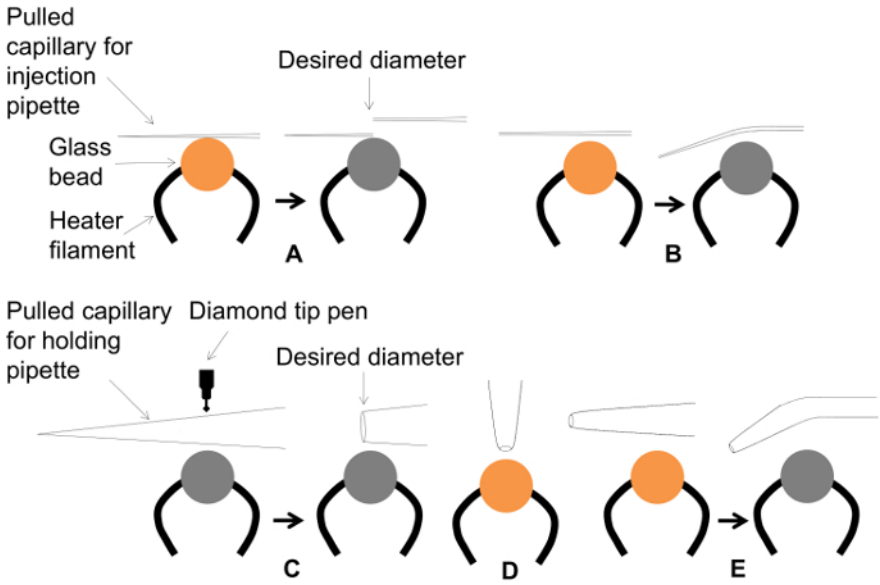

Figure 1: A General Diagram Showing How to Make the Holding and Injection Pipettes. A-B) Injection pipette, **C-E)** Holding pipette. **A)** Gently touch the filament with the pulled pipette at the desired diameter (5 µm). Activate the heater briefly (showed in orange). This will slightly melt the pipette so it adheres to the filament and upon cooling, the pipette will break at the contact point generating a straight cut. **B)** Make and angle in the pipette about 0.5 cm away from its tip by positioning the pipette about 10 µm away from the heater filament. Continue heating until the desired angle is achieved. **C)** Use a diamond tip pen to mark and cut the holding pipette at the desired diameter (180 µm). **D)** In a vertical position, fire-polish the tip of the holding pipette until it reaches the desired internal diameter (40 µm). **E)** Make an angle on the pipette bringing the pipette back to a horizontal positioning a couple µm away from the filament and about 0.5 cm away from its tip. Activate the heater to bend the pipette over the filament. Continue heating until the desired angle is achieved. See [8,9,10] for more details on how to make needles. Please click here to view a larger version of this figure.

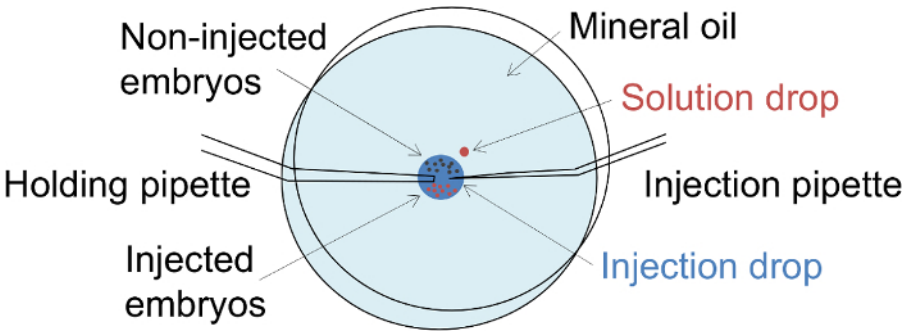

Figure 2: General Setup of Injection Dish. Arrangement of pipettes, drops, and embryos are displayed in this drawing. Please click here to view a larger version of this figure.

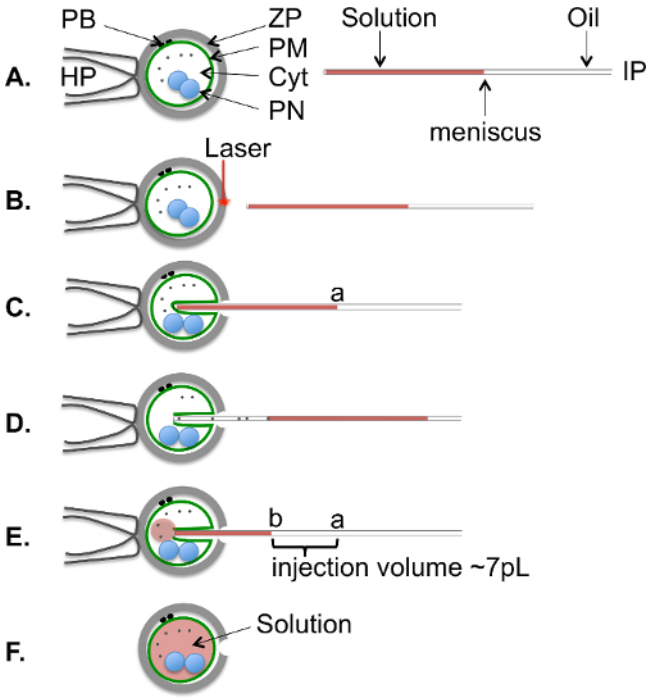

Figure 3: Laser-assisted Intracytoplasmic Injection Protocol. A) A general diagram of a zygote attached to the holding pipette before injection. PB: Polar bodies, ZP: zona pellucida, Cyt: Cytoplasm, PN: pronuclei, PM: Plasma membrane, HP: holding pipette, IP: injection pipette. **B-E)** Microinjection steps: **B)** Make a hole in the ZP of a zygote attached to the holding pipette using a laser. **C)** Introduce the injection pipette towards the opposite side of the embryo. Verify position of meniscus (a). **D)** Break the plasma membrane by aspirating cytoplasmic content inside the injection pipette. **E)** Inject back cytoplasmic content and solution into the zygotic cytoplasm, until the meniscus reaches one zygote diameter (b) past the starting point. Distance a-b represents ~ 7 pl of injected solution. **F)** A general diagram of a zygote after injection. Note that the injected solution homogeneously spreads into the cytoplasm. Please click here to view a larger version of this figure.

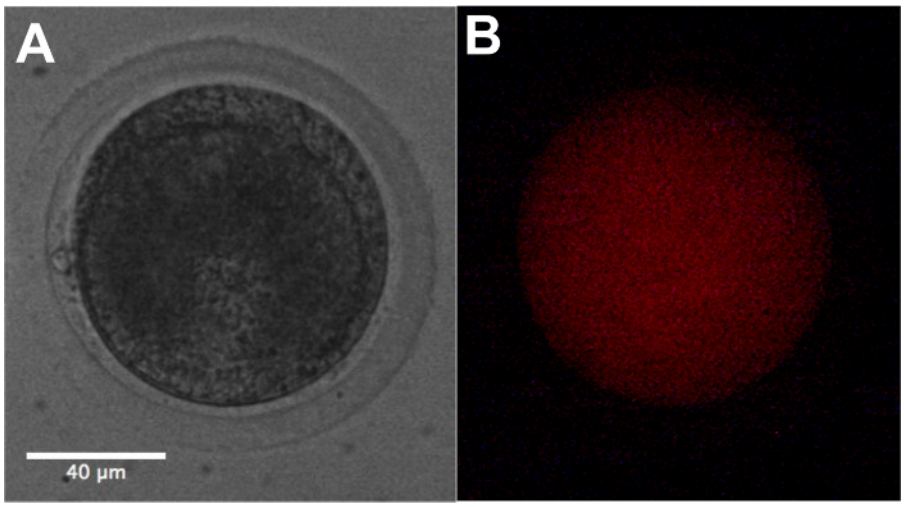

Figure 4: Representative Figure of an Injected Zygote with Dextran-red. A) Bright field image of the injected zygote **B)** Fluorescent image of the injected zygote. Please click here to view a larger version of this figure.

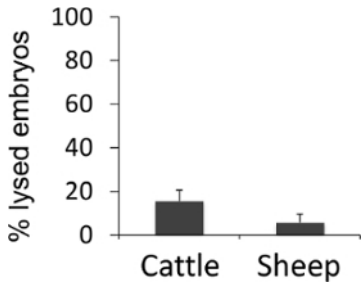

Figure 5: Proportion of Lysed Embryos after Zygote Microinjection in Two Different Species. Data represents 4 replicates for the bovine embryos (total of 103 injected zygotes) and 3 replicates for the ovine embryos (total of 173 injected zygotes). Error bars represent s.e.m. Please click here to view a larger version of this figure.

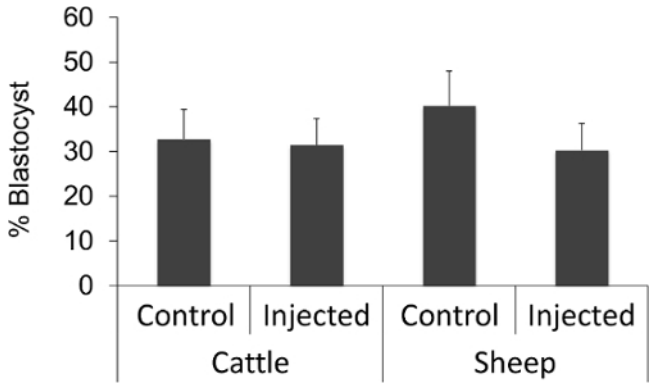

Figure 6: Blastocyst Rates in Bovine and Ovine Embryos. Data represents 4 replicates for the bovine species with a total of 102 injected and 156 control embryos and 3 replicates for the ovine species with a total of 163 injected and 239 control embryos. Error bars represent s.e.m. Please click here to view a larger version of this figure.

Discussion

Microinjection of zygotes is a well-established method for introducing solutions into mammalian embryos. With some variations dependent on the species and the aim of the experiment, this technique can be broadly used. We show how to perform intracytoplasmic microinjection using a laser to assist the entrance of a blunt-end micropipette. Zygotes of some livestock species (such as cattle, sheep, and pig) have a dark cytoplasm, hindering the visualization of the injection pipette once inside the embryo. Also, their plasma membranes are very elastic, making their penetration with a beveled spiked needle (usually used to inject zygotes of rodent species) hard to achieve. To overcome these limitations, we used a laser to enable the passage of a blunt-end needle through the zona pellucida and the subsequent aspiration of cytoplasmic content to ensure breakage of the plasma membrane and the release of the solution inside the cytoplasm of the embryo. Moreover, by injecting in the opposite site of the needle entrance (about 3/4 inside the embryo) we aim to displace the PN and thus avoid their aspiration/injection. Furthermore, this provides more point of contact for the broken membrane to heal, thus resulting in lower lysis rates.

Producing good micropipettes (specially the injecting pipette) and appropriately setting them into the micromanipulator is key to achieving good results with this technique. The injection needle can be used for as long as the injection solution moves smoothly inside the needle. Sometimes, cytoplasmic content or even pronuclei content gets stuck inside the tip of the needle, causing the flow to run unevenly and complicating injection (this also increases lysis rates and delays the process). Replacing the injection needle when this happens is necessary to obtain optimal results with this protocol. The amount of time that the embryos are outside of the incubator is crucial to get consistent survival rates and should not exceed 30 min. After training and practice, operators usually achieve an injection speed of 1-2 injected embryos per minute. Another key point for the success of this protocol is to consistently inject the same amount of volume of solution per embryo. This is easily and accurately controlled by observing the displacement of the solution-oil interface meniscus. It is important that the pipette diameter is consistent between manipulations and that the tip has a regular and constant diameter. With 5 µM ID pipettes at the tip, a 7 - 10 pl of injection volume is achieved by injecting the equivalent to a zygote's length. Over-injection often results in embryo lysis.

Using this protocol, 100% of the embryos are injected properly into the cytoplasm, completely avoiding false perivitelline space injections (**Figure 4**). This maximizes the reliability and reproducibility of the assay being done (regardless of the injected solution) since the results are due only to the effect of the injected solution and not to injection inconsistencies. Some of the injected zygotes will usually lyse due to mechanical damage during injection. A usual rate of survival after injection is 75%[11]. Using this method, we are able to get minimal rates of lysed embryos (**Figure 5**). Also, blastocyst development rates were comparable to non-injected (control) embryos (**Figure 6**), denoting that the injection technique has no detrimental effects on early embryo development. The efficiency of this protocol was recently compared to the conventional cytoplasmic microinjection protocol (direct cytoplasmic microinjection using a beveled spiked glass needle without the use of the laser to penetrate de ZP) for injecting bovine zygotes[5]. The results showed significantly higher rates of lysed embryos and lower rates of blastocysts formation for the direct cytoplasmic microinjection. We believe these differences are due to less mechanical damage during needle penetration when using the laser-assisted cytoplasmic microinjection, making this protocol the preferred one for injecting livestock species in our laboratory.

Here, we present data for bovine and ovine embryos obtained by *in-vitro* fertilization, but have also tested the protocol using *in-vivo* and *in-vitro* swine embryos obtaining similar results (data not shown). Depending on the injected solution, this protocol can be used for many applications. Recently, we have used it to introduce a CRISPR/Cas9 system to knock-out specific genes on *in-vitro* fertilized bovine embryos and found a high proportion of sequenced blastocysts with mutations: 50% of the embryos (n= 6) had biallelic mutations, 33% (n= 4) had monoallelic mutations, and 17% (n= 2) were wild-type [5]. This protocol has been very reliable and could be used in several species and myriad applications.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Work related to this technique is supported by NIH/NICHD RO1 HD070044 and USDA/NIFA Hatch projects W-3171 and W-2112.

References

1. McEvoy, T., Coull, G., Broadbent, P., Hutchinson, J., Speake, B. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *J Reprod Fertil.* **118** (1), 163-170 (2000).
2. Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K., & Palmiter, R. D. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci U S A.* **82**(13), 4438-4442 (1985).
3. Ross, P. J., *et al.* Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. *BMC Dev Biol.* **8** (1), 16 (2008).
4. Canovas, J., Cibelli, J.B., Ross, P.J. Jumonji domain-containing protein 3 regulates histone 3 lysine 27 methylation during bovine preimplantation development. *Proc Natl Acad Sci U S A.* **109**(7), 2400-2405 (2012).
5. Bogliotti, Y. S., *et al.* 4 Developmental outcomes and effiency of two CRISPR/Cas9 microinjection methods in bovine zygotes. *Reprod Fertil Dev.* **27** (1), 94-94 (2015).
6. Bakhtari, A., Ross, P.J. DPPA3 prevents cytosine hydroxymethylation of the maternal pronucleus and is required for normal development in bovine embryos. *Epigenetics.* **9**(9), 1271-1279 (2014).
7. Yaul, M., Bhatti, R., Lawrence, S. Evaluating the process of polishing borosilicate glass capillaries used for fabrication of in-vitro fertilization (iVF) micro-pipettes. *Biomed Microdevices.* ***10*** (1), 123-128 (2008).
8. Sutter Instrument. *Pipette Cookbook 2015 P-97 & P-1000 Micropipette Pullers.* Sutter Instrument, www.sutter.com/PDFs/pipette_cookbook.pdf (2015).
9. Sutter Instrument. *P-97 Flaming/BrownTM Micropipette Puller Operation Manual Rev.* 2.30 - DOM (20140825). www.sutter.com/manuals/P-97-DOM_OpMan.pdf (2014).
10. Cibelli, J.B., Lanza, R.P., Campbell, K.H.S., West, M.D. *Principles of cloning.* Academic Press. (2002).
11. Wang, B. *et al.* Expression of a reporter gene after microinjection of mammalian artificial chromosomes into pronuclei of bovine zygotes. *Mol Reprod Dev.* **60**(4), 433-8 (2001).

