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Transgene Delivery via Microelectromechanical Systems

Aubrey M. Wilson

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Master of Science

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## ABSTRACT

### Transgene Delivery via Microelectromechanical Systems

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Master of Science

The invention of pronuclear microinjection initiated the field of transgenic research. Over 30 years later microinjection remains the most straight-forward and most commonly used transgene delivery option. In this work we address the current progress of microelectromechanical systems (MEMS) used as transgenic delivery mechanisms. The nanoinjector is a specially designed MEMS device which uses electrostatic charge to manipulate transgene molecules. The process of nanoinjection was designed as an alternative to microinjection which causes less damage to developing embryos, improves embryo survival, birth rates, and overall efficiency of injections. In vivo testing of nanoinjection demonstrates it is both safe and effective. Additionally nanoinjection has the potential to make transgenesis via yeast artificial chromosomes more practical as the nanoinjector may prevent shearing of the YAC molecules.

A second nanoinjection protocol termed intracellular electroporetic nanoinjection (IEN) was designed to allow for cytoplasmic injections. Cytoplasmic injections are faster and easier than pronuclear injection and do not require the pronuclei to be visible; yet previous attempts to develop cytoplasmic injection have met with limited success. In IEN injections the nanoinjector is used to place transgenic molecules in the cytoplasm. The transgenes are then propelled through the cytoplasm and electroporated into the pronucleus using electrical pulses. Electroporation of whole embryos has not resulted in transgenic animals, but the MEMS device allows localized electroporation to occur within the cytoplasm, giving transgene access to the pronucleus before degradation can occur. In this report we describe the principles which allow for localized electroporation of the pronuclei including: the location of the pronuclei between 21-28 hours post-hCG treatment, modeling data predicting the voltages needed for localized electroporation of pronuclei, and data on the movement of transgenic DNA based on the voltages delivered by IEN. We further report results of an IEN versus microinjection comparative study in which IEN produced transgenic pups with viability, transgene integration, and expression rates statistically comparable to microinjection. The ability to perform injections without visualizing or puncturing the pronuclei will widely benefit transgenic research, and will be particularly advantageous for the production of transgenic animals with embryos exhibiting reduced pronuclear visibility.

Keywords: nanoinjection, microinjection, transgenic, electroporation, DNA transfer

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A large number of people contributed significantly to the research that went into this project. The students of the Brigham Young University Department of Molecular & Microbiology Burnett Research Laboratory, the students of the Brigham Young University Department of Mechanical Engineering Compliant Mechanisms Research Group (CMR) and the professionals at University of Utah Transgenic and Gene Targeting Mouse Core all played integral roles in the success of this research.

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## CHAPTER 1. INTRODUCTION

### 1.1 Transgenics

In 1980 a ground breaking recombinant DNA technology, pronuclear microinjection, was first reported (Gordon et al. 1980). The report demonstrated that introduction of novel DNA segments to an organism's genome through direct injection was possible. Those DNA segments could be retained, expressed, and passed on not only to subsequent cells during the organism's development, but to its offspring as well. The development of microinjection brought with it the promise of understanding human physiology by teasing apart complex mechanisms one gene at a time. The genetic contributions of disease could finally be defined and new therapies tested in vivo. While cell culture will continue to be a critical tool, ex vivo experiments are invaluable due to the genetic and physiological complexities of living organisms (Doyle et al.). Indeed, microinjection has been used to produce transgenic animals that have delivered significant insights into all fields of biology from basic research to applied medicine (Doyle et al.).

New technologies such as low-cost whole-genome sequencing and genome wide association studies have only added to the number of genes of interest for which a transgenic model is desired (Aitman et al. 2011). The importance of transgenic animals is highlighted by the creation of investigator-driven consortiums which aim to develop transgenic lines for every gene of the mouse genome (Doyle et al.). Increasing understanding of the complexities of disease underscore the need for the next generation of model organisms to more closely resemble actual disease states, and their complex genetic architecture. Furthermore, better models require the use of the most relevant species which are increasingly likely to be non-murine species. The

continued progress of biological research will demand new and innovative techniques for the production of transgenic animals.

## **1.2 Microinjection**

The production of transgenic animals requires foreign DNA to become incorporated into an organism's genome. Various transgene delivery methods have been developed to facilitate the integration of transgene. The simplest method is direct delivery of naked DNA into the pronucleus of a developing embryo through microinjection. The remaining indirect delivery techniques require an intermediate or vector to deliver the transgene to the host such as embryonic stem cells, a viral vector, or an altered nucleus.

Likely due to its simplicity, microinjection is the most commonly used transgene delivery strategy. In this process, fertilized zygotes are harvested from female donors after a series of hormone injections induces super ovulation (Doyle et al.). Timing of harvest and injections is critical as microinjection must occur before the first cell division takes place in order for the transgene to be incorporated into every cell of the resulting organism (Gordon et al. 1980). This leaves just a short window after fertilization occurs and before the pronuclei begin to break down in which injections can be successful. Approximately two picoliters of transgenic solution is injected into one of the two pronuclei in each embryo (Nagy et al. 2003). The pronucleus can be seen to swell slightly during injection which is used as visual confirmation that injection was successful. Injecting transgenic solution into the pronucleus is traumatic for the embryo; some embryos will sustain chromosomal damage and occasionally injections cause the pronucleus to burst (Yamauchi et al. 2007). After injection, surviving zygotes are implanted into pseudo pregnant surrogates and development continues as normal with pups born after ~20 days (Doyle



et al.). If successful, the entire process from hormone injections to birth of fully transgenic animals requires less than one month making microinjection an appealing method for transgenesis.

Microinjection is limited, however, by its low efficiency. Due to the stress of injections a high percentage of microinjected embryos do not survive to birth. Numerous factors influence survival rates which have been reported to fluctuate between 9.4 and 23% of injected embryos (Brinster et al. 1985). Furthermore, once the transgene has been injected into the pronucleus it may or may not become incorporated into the organism's genome through the process of random nonhomologous recombination. (Homologous recombination events also occur, but very rarely, with one homologous recombination occurring for every  $10^3$ - $10^4$  nonhomologous integration events (Brinster et al. 1989; Wall 2001)). Microinjection results in approximately 10-20% integration positive pups (Wall 2001). Expression rates will be somewhat lower than integration rates dependent upon cassette design, degradation before integration, and the location of integration. Despite these limitations, microinjection's speed and simplicity make it a favorable method for producing transgenic animals.

## CHAPTER 2. NANOINJECTION

### 2.1 Introduction

Nano injection is an original transgene delivery technology developed at Brigham Young University as an alternative to microinjection (Aten et al. 2012). While still a form of pronuclear injection, nano injection was designed to causes less damage to developing embryos than microinjection, improve embryo survival, birth rates, and overall efficiency of injections. The major advancement of nano injection is the specially designed microelectromechanical system (MEMS) or nano injector which replaces the hollow needle and fluid pump used for microinjection (See figure 2.1). The MEMS device consists of an electrically conductive lance attached to a compliant mechanism. The MEMS device allows the charge of the lance, and therefore DNA's affinity for it, to be controlled with the flick of a switch. In this new technique, transgene is electrically accumulated on the surface of the lance by applying a positive charge (David et al. 2010). The lance then pierces the zygote's pronucleus with its DNA-coated tip. The DNA is released into the pronucleus by reversing the charge on the lance, and the lance is withdrawn.

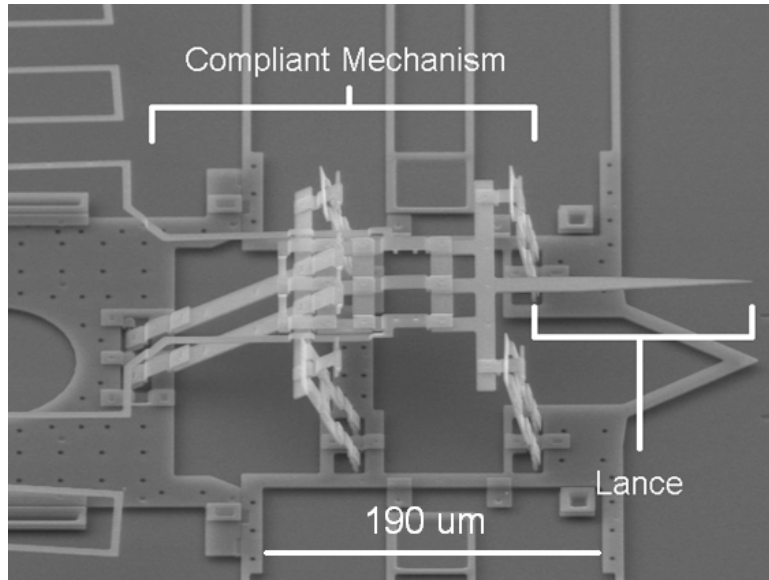


Figure 2.1 A scanning electron micrograph of the MEMS nanoinjector used to perform both nanoinjections and IEN injections. The device consists of an electrically conductive lance to attract, hold, or repel transgene, and a compliant mechanism (Howell 2001) to provide the mobility required for injections.

Using DNA's inherent negative charge to deliver transgene is beneficial in two ways. First, it allows a smaller solid lance ( $0.06 \mu\text{m}^2$ ) to replace a hollow microinjection needle ( $0.78 \mu\text{m}^2$ ) (Aten et al. 2012). This decreases the physical damage sustained by cell and nuclear membranes during injections and reduces the resulting electrolyte loss (Miller et al. 1984). Second, only transgene is accumulated on the lance and then injected into the pronucleus. This mass of the transgene is insignificant compared to the volume of transgenic solution injected during microinjections. Eliminating the extra volume of buffer injected into the pronucleus reduces the strain from pronuclear swelling, the risk of bursting, and the chance of chromosomal damage (Yamauchi et al. 2007). Together these characteristics make nanoinjection a much less traumatic process than microinjection.

## 2.2 Study of Nanoinjection Effectiveness

We designed an experiment which directly compared nanoinjection and microinjection to test the hypothesis that nanoinjection would improve embryo survival (Aten et al. 2012). Mouse embryos were injected with an eGFP containing transgene through either nanoinjection or microinjection and implanted into surrogates. The embryo survival was recorded at the 2-cell stage before implantation and at birth. The resulting pups were then analyzed for eGFP integration and expression. PCR was performed on genomic DNA samples using primers designed for eGFP to test for integration. The PCR results were further confirmed by sequencing and southern blot of a representative sample of individuals. Flow cytometry was performed on peritoneal exudate cells (PEC's), blood, thigh muscle, brain and gut samples to test for eGFP expression. Mice were considered integration positive if PCR successfully amplified eGFP transgene. Expression positive mice were those that were PCR positive and showed eGFP expression in at least one tissue sample.

Nanoinjection and microinjection yielded similar rates of integration per pup ( $17.3 \pm 6.2\%$  and  $14.1 \pm 7.4\%$  respectively) (Aten et al. 2012). Integration positive rates per injected embryo, however, were significantly higher for nanoinjection ( $6.7 \pm 2.5\%$ ), than microinjection ( $1.9 \pm 0.8\%$ ) (See figure 2.2) (Aten et al. 2012). Nanoinjected embryos were more likely to survive the shock of injection and initial 24 hours of culturing as well as survive through gestation (Aten et al. 2012). These factors lead to significantly more births and transgenic pups in the nanoinjection group. Only 13% of the embryos injected through standard microinjection survived to birth while nanoinjection rates were above 40% (Sumiyama et al. 2010; Aten et al. 2012). For this experiment the odds ratio for producing a transgenic animal through

nanoinjection was 4.2 times higher than through microinjection (Aten et al. 2012). Nanoinjection proved to be a more efficient process than microinjection requiring fewer embryos, injections, and surgeries to produce an equivalent number of transgenic animals. As a technique nanoinjection has the potential to significantly reduce the cost of producing transgenic animals. Its efficiency also decreases the number of animals which must be sacrificed in the process of producing a transgenic animal which is in agreement with The Three R's guideline of reduction (reduce, replace, refine) (Russell 1995). This reduction could arguably make nanoinjection a more ethical option for transgene delivery than microinjection.

A. Data on embryo viability, birth rate per viable embryo, and the number of pups with positive integration and expression as observed in the nanoinjection-versus-microinjection comparison study.

	<b>2-cell Embryos /Injected Embryo<sup>†</sup></b>	<b>Births /Transferred Embryo<sup>†</sup></b>	<b>Integration Positive / Pup<sup>a</sup></b>	<b>Expression Positive / Pup<sup>a</sup></b>
<b>Nanoinjection</b>	288/371 (77.6%)	151/288 (52.4%)	23/140 (16.4%)	13/140 (9.3%)
<b>Microinjection</b>	351/642 (54.7%)	81/339 (23.9%)	10/81 (12.3%)	6/81 (7.4%)

B. Data from the nanoinjection-versus-microinjection comparison study reported per injected zygote.

	<b>2-cell Embryos /Injected Embryo<sup>†</sup></b>	<b>Births /Injected Embryo<sup>†b</sup></b>	<b>Integration Positive / Injected Embryo<sup>†b</sup></b>	<b>Expression Positive / Injected Embryo<sup>†b</sup></b>
<b>Nanoinjection</b>	288/371 (77.6%)	151/371 (40.7%)	23/371 (6.2%)	13/371 (3.5%)
<b>Microinjection</b>	351/642 (54.7%)	81/630 (12.9%)	10/630 (1.6%)	6/630 (1.0%)

Table 2.2 Results of the nanoinjection vs. microinjection comparative study. See Aten et al. 2012.

† Results are statistically significant between nanoinjected and microinjected groups with  $p < 0.001$

+ Results are statistically significant between nanoinjected and microinjected groups with  $p < 0.01$

<sup>a</sup> 11 pups from the nanoinjection group were abandoned by their mother shortly after birth and were not analyzed

<sup>b</sup> 12 microinjected 2-cell embryos lost during the transfer procedure were not included in this dataset

## CHAPTER 3. YEAST ARTIFICIAL CHROMOSOME NANODIJECTION

### 3.1 Yeast Artificial Chromosomes

The coding capacity of yeast artificial chromosomes (YACs) have the potential to increase cassette size and improve fidelity of transgene expression in transgenic animals (Schedl et al. 1993). Transgene preparations are generally constructed and maintained using plasmids. Plasmids limit the overall size of the insert and the number of introns, genes, and regulatory regions that can be included (Houdebine 1997). Other vectors such as YACs, BACs and PACs have the potential to increase this coding capacity and the complexity of cassettes that could be designed (Ohtsuka et al. 2010). YACs can stably maintain over 1000kb which would allow complex transcriptional units or gene clusters to be included along with regulatory units responsible for appropriate expression (Moreira et al. 2004). The YACs could also be designed to shield the transgene from the effects of DNA surrounding the integration site which can potentially cause weak, stochastic, or ectopic expression.

YACs can be introduced directly into embryos through standard microinjection. Other delivery methods such as spheroplast fusion and lipofection have also been demonstrated (Schedl et al. 1993; Houdebine 1997). Integration rates in offspring vary between 5-20%, although only 20-71% (Houdebine 1997) of those individuals retain the entire integrated YAC (Moreira et al. 2004). Integrity can be assessed through PCR to detect presence of left and right vector arms.

YACs have some other major advantages originating from their yeast hosts. Yeast have an extremely robust system of homologous recombination making modification of YACs straightforward and efficient (Schedl et al. 1993). Homologous recombination events predominate in this system allowing any conceivable cassette to be constructed using standard

protocols (Giraldo and Montoliu 2001). Amplification of DNA in a yeast system is also efficient with the addition of a conditional centromere. After amplification YACs can consist of up to 50% of the yeast's genomic material (Schedl et al. 1993).

While modification and amplification of YACs are relatively straight forward processes, the purification of YACs prior to injection is a tedious and time consuming procedure requiring materials and expertise not present in every laboratory. YAC purifications must be of the correct concentration as low yields are ineffective and high concentrations can cause embryo death (Houdebine 1997). Purification must also be relatively free of impurities that can also prove fatal to embryos. Special precautions must be taken throughout the process to prevent shearing the large molecule into fragments (Schedl et al. 1993). YACs must be maintained in high protein buffer at all times to encourage the molecule to remain tightly coiled and prevent breakage (Houdebine 1997). Pipet tips with larger than usual diameters are always used. One concern when microinjecting YACs is the size of the microinjection needle which, of necessity, is extremely small. Despite the potential of YACs as transgenic vectors they are rarely used in transgenesis by the majority of laboratories due in part to these concerns. However, those laboratories that have chosen to focus on YACs as transgenic vectors have had a great deal of success

### **3.2 In vivo YAC Nanoinjection**

During nanoinjections the nanoinjector holds transgene molecules to the outside of the lance instead of forcing it through a hollow needle. This means nanoinjection has a much higher upper limit on the size of the transgene that can be injected without shearing. Nanoinjection of YACs in place of microinjection could improve transgenic yields as the risk of shearing the YAC



molecule would be reduced. To test this theory, we designed a comparative study of the effectiveness of nanoinjection and microinjection of YACs.

The yeast artificial chromosome YRT3 was obtained from the lab of Dr. Lluís Montoliu which is designed to convert the coat color of an albino mouse into an agouti brown coat color. Four days were reserved at the Transgenic and Gene Targeting Center at the University of Utah to compare nanoinjection and microinjection using the YRT3 YACs. Unfortunately, the core did not have the appropriate age of albino mice needed to serve as embryo donors at the time of our scheduled experiment. Older female mice (1-3 months) were substituted and this age difference resulted in significantly lower yields of fertilized embryos than expected. As a result of the low embryo yield, we were forced to perform control group microinjections on only one day in four to allow more of the eggs to be directed to the nanoinjection group. Only on day 2 was the embryo harvest sufficient to allow any embryos to be microinjected. Over the four day experiment, 234 embryos were nanoinjected and 67 embryos were microinjected. This ultimately resulted in 54 nanoinjected pups (one of which died after birth) and 11 microinjection pups.

The pups' coat color was scrutinized at weaning, but none of the surviving 64 pups had any agouti coloring (Table 3.1). Tail snips were taken and genomic DNA isolated to allow for genotypic analysis and detection of partial YAC integrations. Five different primer sets were selected which correspond to different regions of the YAC YRT3. After optimization and testing of primers, three sets (Trp1, Ade2, and Leu2) were chosen to test the YAC pups. All individuals were tested for integration using the three YAC-specific primers and beta-actin as a control for DNA purity. Only one individual, #9, showed evidence of partial integration after amplification with the Trp1 primer set. This pup came from the microinjection group from day 2 of the experiment.

There are multiple possibilities based on the results of this experiment. First, we must concede that it is possible that nanoinjection does not effectively deliver YAC molecules to the pronucleus. A second possibility is that the quality of the embryos derived from older females and used in this experiment confounded the results and prevented the production of transgenic animals. A third possibility, and perhaps the most compelling, is the total number of embryos was too low to expect the production of a YAC integration. The integration rate for microinjection of YACs and the expected integration rate using nanoinjection are low. Lluís indicated that an experiment should include more than 100 pups born to anticipate a single YAC positive integrant. In the case of this study, only 1 of 63 pups integrated a portion of the YAC. Clearly, a larger number of pups must be produced to obtain data on the efficacy of nanoinjection of YACs. The results of this experiment ultimately are inconclusive and further testing is required to evaluate the potential of nanoinjection in YAC transgenesis.

A. Data on embryo viability, birth rate per viable embryo, and the number of pups with positive YAC integration and expression as observed in the nanoinjection-versus-microinjection comparison study.

	<b>2-cell Embryos /Injected Embryo</b>	<b>Births /Transferred Embryo</b>	<b>Integration Positive / Pup</b>	<b>Expression Positive / Pup</b>
<b>Nanoinjection</b>	195/234 (83.3%)	52/195 (26.7%)	0/52 (0.0%)	0/52 (0.0%)
<b>Microinjection</b>	41/67 (61.2%)	11/41 (26.8%)	1/11 (9.1%)	0/11 (0.0%)

B. Data from the YAC nanoinjection-versus-microinjection comparison study reported per injected zygote.

	<b>2-cell Embryos /Injected Embryo</b>	<b>Births /Injected Embryo</b>	<b>Integration Positive / Injected Embryo</b>	<b>Expression Positive / Injected Embryo</b>
<b>Nanoinjection</b>	195/234 (83.3%)	52/234 (22.2%)	0/234 (0.0%)	0/234 (0.0%)
<b>Microinjection</b>	41/67 (61.2%)	11/67 (16.4%)	1/67 (1.5%)	0/67 (0.0%)

Table 3.1 Results of the in vivo YAC experiment. No expressing pups were produced in this experiment. The microinjection control group produced one partial integration positive pup while the nanoinjection group did not produce any integration positive pups. A larger study is required to determine the efficacy of YAC nanoinjection.

## CHAPTER 4. INTRACELLULAR ELECTROPORETIC NANOINJECTION

### 4.1 Cytoplasmic Delivery

Both microinjection and nanoinjection are examples of pronuclear delivery as the transgene is delivered directly through puncturing the pronucleus. A technique which would allow transgene integration after injection into the cytoplasm has been an elusive goal for decades. The possibility of producing transgenic animals through cytoplasmic microinjection was first explored shortly after development of pronuclear microinjection, but it failed to produce transgenic offspring (Brinster et al. 1985). The transgene was either degraded in the cytoplasm before nuclear transport could occur or lacked signals necessary for transport altogether. Attempts have been made to stabilize foreign DNA by encapsulation in liposome and thereby enable integration, but have also failed to produce transgenic animals (Loskutoff et al. 1986; Kraemer 1988). One report described the successful use of poly-L-lysine bound transgene which allowed for transgene integration (Page et al. 1995). The poly-L-lysine contribution to integration was unknown, but both increased stability and nuclear uptake are thought to be involved. Although a common technique for transfection this is the only mention of poly-L-lysine used in transgenesis that the authors could find (Hartono et al. 2012; Kim 2012). We are unaware if this technology is still being pursued or if its complexity has prevented its adoption.

An additional hurdle to the development of cytoplasmic delivery strategies is late integration. Some techniques could potentially allow integration to occur only after pronuclear membranes breakdown and the genome has been replicated in preparation for cell division. If integration occurs after replication the transgene will only be present in half of the daughter cells. Integration events occurring after multiple cell divisions could also be possible producing mice

with only 1/4th or 1/16<sup>th</sup> of their cells containing the transgene. When late integration event occur transgene may not be present in the germ line of these mice in which case they will not produce transgenic offspring.

Despite the challenges, the advantages of cytoplasmic injection make it an appealing topic of study. Injections into the cytoplasm are easier than injections into a much smaller pronucleus even under ideal conditions. They can be performed faster than pronuclear injections as the embryo does not have to be carefully positioned for good line of sight. Also the pronucleus is not physically punctured reducing the strain of injections on the developing embryo. Difficult pronuclear injections such as those in strains of mice which have smaller than average pronuclei, or in species which lack transparent embryos would not pose a problem for cytoplasmic injections (Wall 2001; Osman et al. 1997). The development of an effective cytoplasmic delivery technique could be of great benefit to the field of transgenic research, especially for those working with species for which microinjection is difficult or impractical.

#### **4.2 Intracellular Electroporetic Nanoinjection**

The success of pronuclear nanoinjection encouraged us to investigate other advantages offered by the MEMS nanoinjector including the possibility of cytoplasmic delivery. Electric fields of appropriate magnitude and duration induce electroporation - the formation of transient pores to form in lipid membranes (Tsong 1991). These pores lead to increased uptake of large molecules including DNA. The voltages required for electroporation are also sufficient to induce electric field driven motion, or electrophoresis, of a DNA molecule (David et al. 2011). These events would allow a transgene to be deposited in the cytoplasm and to then be propelled toward the pronuclei and electroporated inside without the pronucleus being punctured or even

visualized. We named this protocol intracellular electroporetic nanoinjection (IEN). We hypothesized that if the embryo could survive the voltages necessary for electroporation IEN could be used to effectively deliver transgene and produce transgenic animals.

Intracellular electroporetic nanoinjection relies on the same MEMS nanoinjector as standard pronuclear nanoinjection described above. The difference in the techniques lies in the intensities of the voltages used to release the transgene from the lance after accumulation. While nanoinjection uses a low negative voltage to simply release the transgene in the pronucleus IEN uses a series of negative pulses with voltage that exceeds the decomposition voltage of the system. These negative pulses simultaneously produce an electroporation envelope which forms pores in the nuclear membrane and causes electric field driven motion of the transgene away from the lance. A fraction of the transgene molecules will be propelled in the direction of one of the pronuclei and enter through the transient pores produced by the electroporation envelope. Once in the pronucleus the transgene may become incorporated into the genome through nonhomologous recombination.

#### **4.2.1 Pronuclear Location and Migration**

It is well documented that fertilization of the mouse oocyte initiates the migration of the male and female pronuclei toward each other to form a single metaphase plate for the first cell division, but the exact location of the pronuclei has not been established (Mayer et al. 2000; Kubiak et al. 2008). In order to design an IEN protocol able to produce an appropriately sized area of effect, it was necessary to determine the location of pronuclei within the embryo during the time period injections would occur. We performed an observational study of healthy fertilized CD1 embryos to determine general embryo architecture including pronuclear size and

location between 21-28 hours post hCG treatment (Figure 4.1). Over 3000 images were taken using confocal microscopy and examined using digital analytical tools<sup>1</sup>. General architecture measurements are presented in table 4.2.

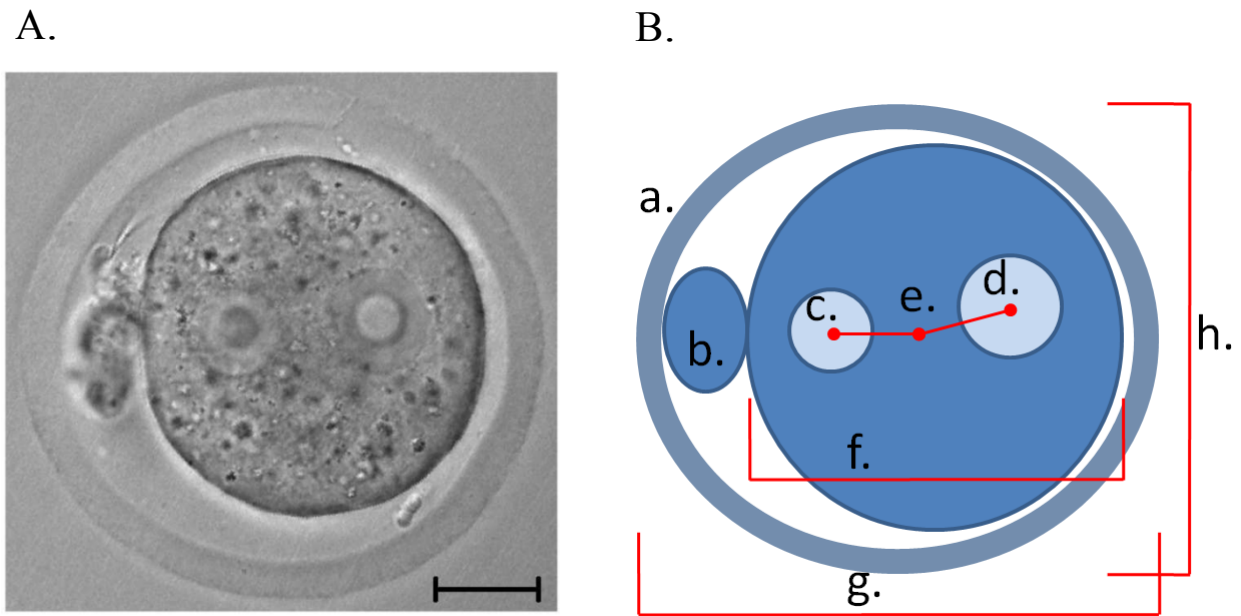


Figure 4.1 Embryo Measurements. A) Example of a DIC of a fertilized mouse embryo. Scalebar represents 20µm. B) Diagram of an embryo. Labeled features include: a. Zona Pellucida b. Polar Body c. Female Pronucleus d. Male pronucleus e. Distance from center of embryo to pronuclei centers f. Cell diameter g. Zona length h. Zona width.

<sup>1</sup> The data and images presented in this section were generously contributed by Justin Black

	<b>Size <math>\mu\text{m}</math></b>
Zona Length	98.3 $\pm$ 5.1
Zona Width	94.6 $\pm$ 4.4
Cell Diameter	69.5 $\pm$ 2.7
Male Pronucleus Diameter	19.3 $\pm$ 1.7
Female Pronucleus Diameter	15.1 $\pm$ 1.3

Table 4.2 Average embryo measurements and one standard deviation of CD1 embryos (n=106). Average sizes did not change significantly between 21-28 hours post hCG injection.

The female pronucleus, defined as the smaller pronucleus and closest to the polar body (Nagy et al. 2003), was found to have a biphasic migration pattern. Between 21-23 hours we observed that the female pronucleus migrates toward the center of the embryo at an average rate of 2.8 $\mu\text{m/hr}$  (P-value=0.029). During this same time span, the distance between male and female pronuclei does not change significantly (P-value=0.097). After 23 hours, the female pronucleus' migration rate towards the center of the embryo slows to 0.9  $\mu\text{m/hr}$  (P-value=0.039). After 23 hours the distance between pronuclei begins to decrease - (P-value=0.047), suggesting that the direction of migration of the female pronucleus shifts from the center of the embryo toward the male pronucleus around 23 hours (Figure 4.3). We did not detect a statistically significant change in the location of the male pronucleus relative to the center of the embryo between 21-28 hours post hCG injections (P-value=0.228 for 21-23 hrs, and P-value=0.503 for 23-28 hrs), suggesting that the male pronucleus completes its migration toward the center prior to 21 hours. The center of the male pronucleus remains 15.95 $\pm$ 5.46  $\mu\text{m}$  from the center of the embryo between 21-28 hours. On average the center of the female pronucleus is located 21.07 $\pm$ 4.51  $\mu\text{m}$  from the



center of the embryo. Based on these results, we concluded that the area of effect of the localized electroporation should extend at least 21  $\mu\text{m}$  from the embryo center to ensure good coverage of the pronuclei throughout the time period in which transgene nanoinjection is performed.

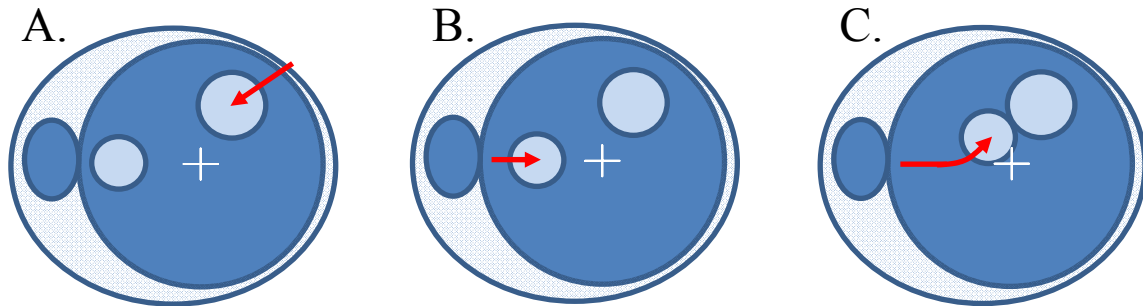


Figure 4.3 Model of Pronuclear Migration in the Mouse Embryo. (A) The male pronucleus migration occurs prior to 21 hrs post hCG. (B) The female pronucleus migrates towards the center of the cell at a rapid pace between 21-23 hrs post hCG. (C) The female pronucleus continues to migrate, but shifts direction to migrate towards the male pronucleus between 23-28 hrs post hCG. The white crosshair represents the cell center in each panel.

#### 4.2.2 Determination of the Electroporation Envelope

When appropriate voltages are applied to cells there is an increase in electrical conductivity and membrane permeability, a process known as electroporation. Careful design of the electroporation pattern is critical to prevent catastrophic cellular damage as a result of excessive voltage or duration (Tsong 1991). The electroporation envelope is calculated as the region of the embryo in which the electric field is greater than or equal to that required to open membrane pores. Based on previous work done on the electroporation of embryos (Grabarek et

al. 2002), 200V/cm was selected as a threshold value for reversible pore formation. Simulations of various voltages were run using a simulation model (David et al. 2010, 2011) running in MATLAB (by Mathworks) to find a voltage whose electroporation envelope around the lance would include both pronuclei<sup>2</sup>. Results of the simulations identified an appropriate repulsion voltage of 2 V above the decomposition voltage (a total of approximately 5.35 volts). The decomposition voltage is empirically determined for each system and represents the voltage at which electrolysis begins (Delgado et al. 2007). Figure 4.4 is the simulation output for the size of the electroporation envelope relative to an embryo and pronuclei when 2 V above decomposition is applied to the lance. Large portions of the membranes of both pronuclei overlap with the electroporation envelope. These regions of the pronuclear membranes should experience transient pore formation giving transgene access to their interior.

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<sup>2</sup> Computer simulation data and images presented in this paper were generously contributed by Nathan Toone

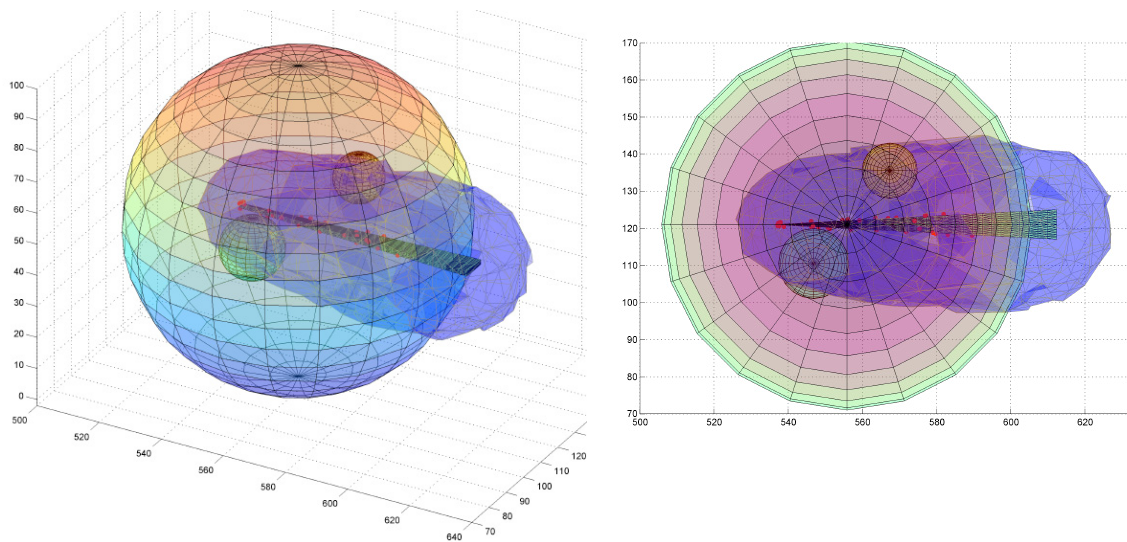


Figure 4.4 Determination of the electroporation envelope area of effect. The electroporation envelope (blue) represents the area of the cell experiencing 200V/cm or greater when 2 volts above decomposition is applied to the lance. The electroporation envelope partially overlaps with both the male pronucleus (green) and female pronucleus (orange) causing pores to form in the nuclear membranes and allowing transgene to enter.

### 4.2.3 Electric Field-Driven Transgene Repulsion

The voltages used to produce the electroporation envelope also induce electric field-driven motion of the transgene away from the lance (David et al. 2010). Although the transgene moves quickly it does not reach a pronucleus instantaneously. The repulsion time must be sufficient for the transgene to travel through the cytoplasm and enter a pronucleus. Computer modeling was used to simulate a 1599 basepair transgene molecule being repelled at 2 volts above decomposition for various durations to identify suitable repulsion times (David et al. 2011). Ultimately the repulsion duration of 5 milliseconds was selected as our starting point

which produced an average repulsion distance of 25.06 $\mu\text{m}$  (figure 4.5). This distance is sufficient for any transgene molecule with the correct trajectory to reach a pronucleus and have a chance to enter through transient pores.

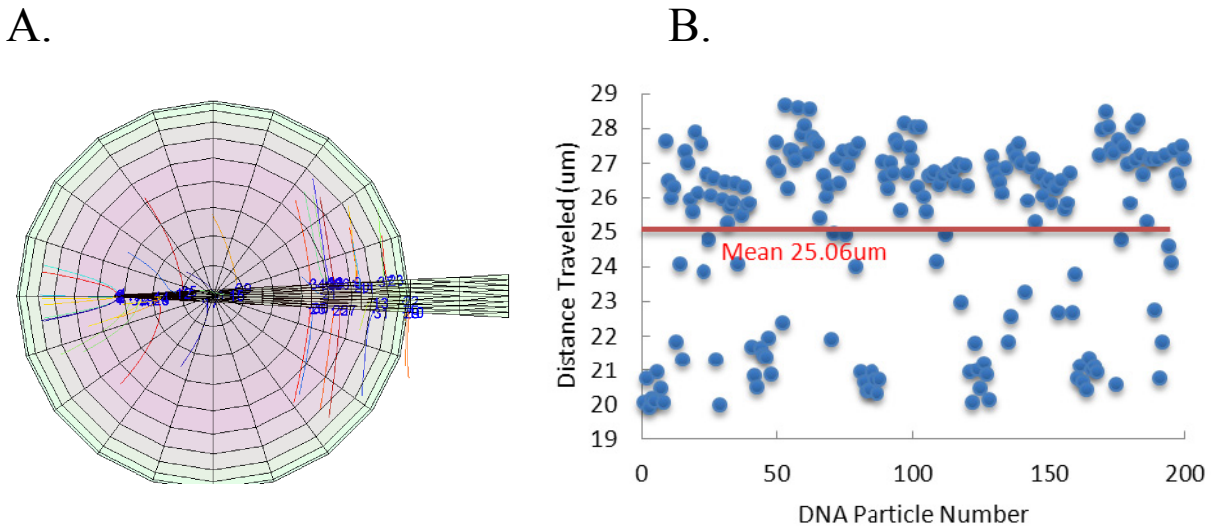


Figure 4.5 Transgene repulsion distance modeling. A) Computer simulation predicts the movement of multiple transgene molecules being repelled from the lance. Only a fraction of transgene molecule would have the correct trajectory to encounter a pronucleus. B) Points on the graph were generated results of 5 simulations each with 40 transgene molecules repelled for 5 milliseconds at 2 volts above decomposition (5.35V). The average repulsion distance obtained was 25.06  $\mu\text{m}$ .

### 4.3 In vivo Testing of IEN Protocols

With the general requirements of IEN estimated through simulations, in vivo experiments were then used to confirm these values and further optimize the protocol. A suitable combination of pulse number, duration, and intensity was sought out. Various injection buffers were tested

along with transgene preparation protocols and injection techniques. Over the next six months we performed 34 small scale studies in which IEN was used to inject between 30-200 embryos with EGFP transgene and were then implanted into surrogates. Progress was monitored by harvesting decidua at 10 days and testing for transgene integration and expression through PCR and fluorescent microscopy respectively. Two pulse patterns showed promise and both were used in large scale studies designed to test effectiveness and allow for comparison to microinjection. The two patterns were arbitrarily designated as pattern 1 and pattern 2 and differed slightly in their grouping of pulses and total repulsion time.

#### **4.3.1 IEN Pattern 1**

To demonstrate the effectiveness of IEN we performed a comparative study using pronuclear microinjection as a control. We injected EGFP transgene into fertilized embryos using either pronuclear microinjection or IEN protocol 1 (10 pulses 0.5 milliseconds in duration at 2 volts above decomposition). All other aspects of embryo harvest, culture, and implantation were identical between the two groups. Injections occurred over the course of two days and included over 600 injected embryos. Viability of injected embryos was recorded 24 hours after injection and at birth to compare survivability of the procedures. After birth and weaning, pups were tested for transgene integration and expression using PCR analysis and flow cytometry respectively to determine transgenic rates. Sequencing of PCR product and southern blot analysis were also performed on a subset of individuals to confirm integration was accurately detected. Survival, integration, and expression data are shown in table 4.6 and figure 4.7. IEN and microinjection survival, integration, and expression rates are not significantly different according to Fisher's exact test confirming that IEN is both safe and effective compared to microinjection.

A. Data on embryo viability, birth rate per viable embryo, and the number of pups with positive integration and expression as observed in the IEN Pattern 1-versus-microinjection comparison study.

	<b>2-cell Embryos /Injected Embryo</b>	<b>Births /Transferred Embryo</b>	<b>Integration Positive / Pup</b>	<b>Expression Positive / Pup</b>
<b>IEN Pattern 1</b>	202/344 (58.7%)	79/202 (39.1%)	21/79 (26.5%)	5/79 (6.3%)
<b>Microinjection</b>	168/295 (56.9%)	55/168 (32.7%)	16/55 (29.1%)	6/55 (10.9%)

B. Data from the IEN Pattern 1-versus-microinjection comparison study reported per injected zygote.

	<b>2-cell Embryos /Injected Embryo</b>	<b>Births /Injected Embryo</b>	<b>Integration Positive / Injected Embryo</b>	<b>Expression Positive / Injected Embryo</b>
<b>IEN Pattern 1</b>	202/344 (58.7%)	79/344 (23.0%)	21/344 (6.1%)	5/344 (1.5%)
<b>Microinjection</b>	168/295 (56.9%)	55/295 (18.6%)	16/295 (5.4%)	6/295 (2.0%)

Table 4.6 Results of IEN pattern 1 in vivo testing and microinjection control. There is no statistical difference between IEN rates and microinjection rates according to Fishers exact test at the .05 level.

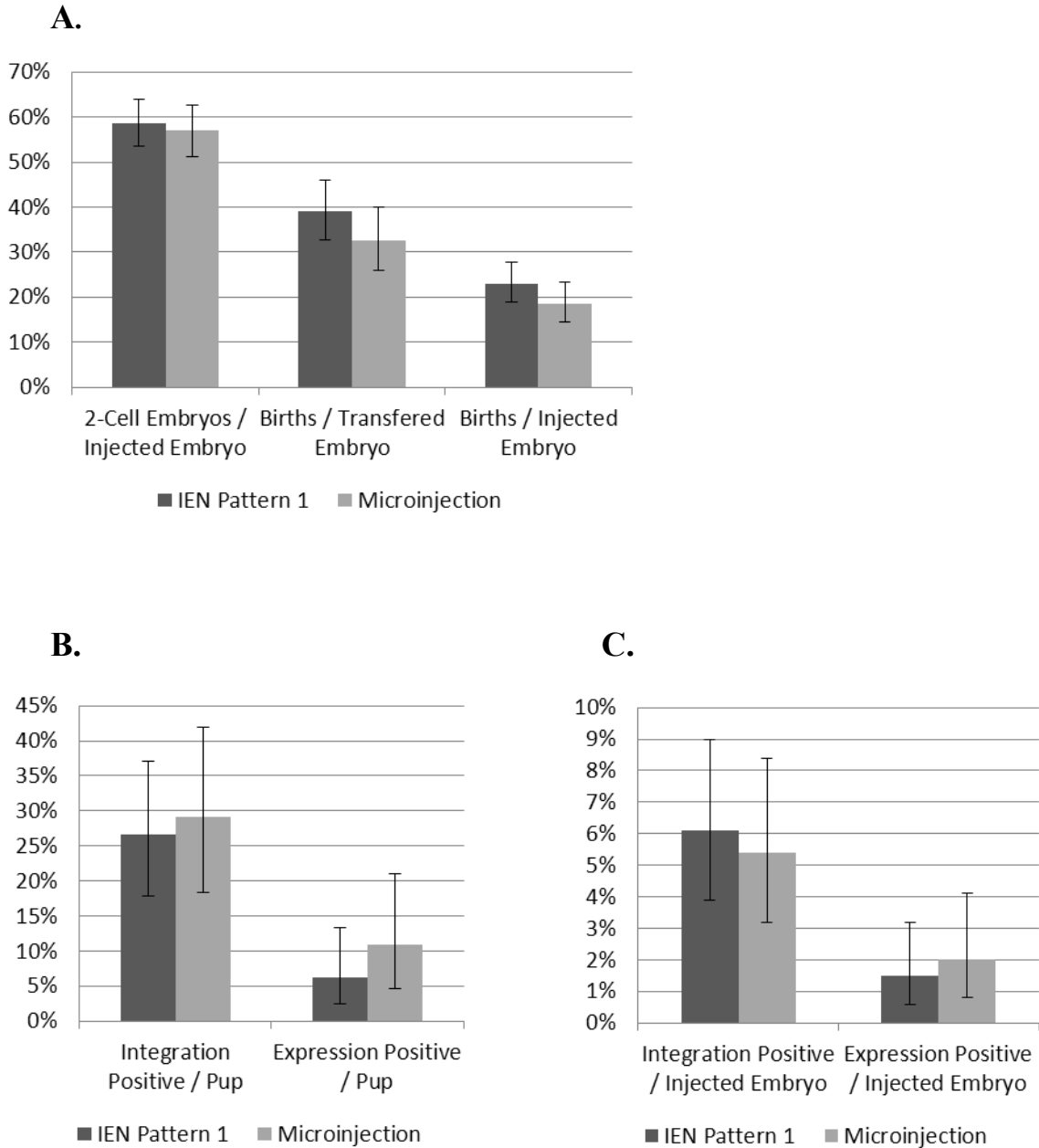


Figure 4.7 Comparison of IEN pattern 1 and microinjection groups' survival, integration, and expression rates. A) Embryo survival rates for IEN and microinjection. B&C) comparison of integration and expression rates per pup and per injection respectively. None of the comparisons shown were statistically different according to Fishers exact test. Plotted confidence intervals are Jefferys 95% confidence intervals for binomial proportions.

IEN is likely a successful delivery strategy because it facilitates nuclear uptake of the transgene and prevents prolonged exposure of the transgene to the cytoplasm thereby preventing enzymatic degradation. Similar rates of IEN pattern 1 and microinjection transgene integration and expression are not surprising as once the transgene arrives in the pronucleus, integration will proceed through the same mechanism of nonhomologous recombination which acts as the rate limiting step for both processes (Brinster et al. 1989). IEN offers a number of advantages over pronuclear injections. Cytoplasmic injections are faster and easier because all injections can be performed identically into the center of the embryo without regard to the location of the pronuclei. This eliminates the time-consuming repositioning required for an ideal pronuclear injection. While the option of performing cytoplasmic injections may be most advantageous for researchers working with species or strains in which microinjection is challenging, simplifying the process of transgenesis will make the production of transgenic models accessible to a greater number of researchers.

Another intriguing possibility which has not yet been pursued is localized electroporation of transgene into other cellular structures such as the mitochondria. Manipulation of the mitochondrial genes through transgenesis could be beneficial for studying metabolic functions and diseases. There is no currently established technique for specific transgenesis of mitochondrial genes in higher organisms. IEN is a revolutionary transgenic technology which has the potential to shift the paradigms of transgene delivery and contribute a great deal to biological research.



### **4.3.2 IEN Pattern 2**

Pattern 2 was also tested for effectiveness in an analogous experiment. Pattern 2 consisted of 2 sets of 7 pulses 0.5 milliseconds in duration at 2 volts above the decomposition voltage. While the 24hr and gestational survival using this protocol were promising none of the resulting pups expressed the transgene (Table 4.8). This lack of expression was likely not a result of the IEN protocol however as the microinjection group also failed to produce any expressing pups during this experiment demonstrating a general failure in the experiment. Microinjection is expected to produce between 10-20% integration positive pups and only slightly lower rates of expression (Wall 2001). The microinjection integration rate of 17.0% is within that range but and expression rate of 0.0% is not. We cannot be certain what aspect of the experiment failed but partial degradation of the transgene prior to integration could explain these results as the same transgene had produced expressing pups in other experiments. Pattern 2 may very well also function as a transgene delivery mechanism under ideal conditions and should be given further consideration.

A. Data on embryo viability, birth rate per viable embryo, and the number of pups with positive integration and expression as observed in the IEN Pattern 2-versus-microinjection comparison study.

	<b>2-cell Embryos /Injected Embryo</b>	<b>Births /Transferred Embryo</b>	<b>Integration Positive / Pup</b>	<b>Expression Positive / Pup</b>
<b>IEN Pattern 2</b>	253/329 (74.6%)	114/253 (45.1%)	11/114 (9.6%)	0/114 (0.0%)
<b>Microinjection</b>	151/260 (58.1%)	47/151 (31.1%)	8/47 (17.0%)	0/47 (0.0%)

B. Data from the IEN Pattern 2-versus-microinjection comparison study reported per injected zygote.

	<b>2-cell Embryos /Injected Embryo</b>	<b>Births /Injected Embryo</b>	<b>Integration Positive / Injected Embryo</b>	<b>Expression Positive / Injected Embryo</b>
<b>IEN Pattern 2</b>	253/329 (74.6%)	114/329 (33.6%)	11/329 (3.2%)	0/329 (0.0%)
<b>Microinjection</b>	151/260 (58.1%)	47/260 (18.1%)	8/260 (3.1%)	0/260 (0.0%)

Table 4.8 Results of IEN pattern 2 in vivo testing and microinjection control. 24 hour and Gestational survival of the IEN group is promising but no expressing pups were produced. The lack of expression positive pups from the microinjection control group suggests a general failure in this experiment.

## CHAPTER 5. MATERIALS AND METHODS

### *Confocal microscopy of embryos*

Harvested embryos were rinsed in M2, rinsed again in PBS, then cultured in KSOM and incubated at 37°C and 5% CO<sub>2</sub> until the time of imaging. Embryos were transferred to a wellled microscope slide, inverted, and placed into position on the microscope platform. Embryos which showed signs of polyspermy, fragmentation of the cytoplasm, or lack of fertilization were discarded. 106 quality embryos were imaged and used to perform the measurements to acquire the data presented. An Olympus Confocal Laser Scanning Biological Microscope FV300 and Fluoview software were used for image acquisition and analysis respectively. Embryos were imaged using the 60x objective.

### *Imaging and analysis of embryos*

Z-stacks were captured in 1.5 or 2  $\mu\text{m}$  increments to produce 3D images. Measurements of the cell membrane (not the zona) were used to identify widest portion of the embryo. The midpoints of diameter in both X and Y planes were used to identify the center of the embryo. All subsequent measurements were taken from that point. Basic geometry formulas were used to determine distances between items not in the same plane. 30-50 images were taken per cell to produced measurements obtained for this report.

### *EGFP transgene preparation*

The pCX-GFP plasmid encodes enhanced green fluorescent protein (EGFP) under the control of the chicken  $\beta$ -actin promoter (CAG). Transgene was prepared for injection by restriction digest with Stu I, and SpeI followed by gel electrophoresis and purification with QIAEX II kit (Qiagen, Valencia, CA). For nanoinjection transgene was diluted to 10-15 ng/ $\mu\text{l}$  in

PBS. For microinjection, the transgene was diluted to a concentration of 2-3 ng/ $\mu$ l in low (0.1M) EDTA TE (pH 7.4).

#### *Mice, zygotes, and embryos*

In vivo mouse work was performed at the University of Utah Transgenic and Gene Targeting Mouse Core in Salt Lake City, Utah. All animal use was in accordance with guidelines of the Animal Welfare Act and followed protocols approved by the Institutional Animal Care and Use Committee (IACUC). Female C57Bl/6J x CBA/J F1 mice were treated with 5 units pregnant mare serum gonadotropin (PMS) (NHPP, Torrance, CA) 3 hrs prior to the dark cycle, 47 hours later treated with 5 units human chorionic gonadotropin (hCG) (Sigma cat #CG-10) and co-housed with stud males. Donor embryos were harvested 18 hours after hCG injection from females with a vaginal plug by dissection of cumulus mass from the oviducts. Cumulus mass was incubated for in 800 units/ml of hyaluronidase (Sigma cat #H4272) in M2 medium (Sigma cat #M7167) for two minutes. Zygotes were rinsed in M2 medium, then maintained in a drop of M16 medium (Sigma cat #M7292) under mineral oil (Sigma cat #M8410) at 37° C and 5% CO<sup>2</sup>.

For pronuclear microinjection and nanoinjection, zygotes with obvious pronuclei were chosen for injection. After injection zygotes were cultured in a 50  $\mu$ l drop of M16 under oil overnight. Healthy two-cell stage embryos were rinsed three times in 100  $\mu$ l drops of M2 and surgically implanted into the oviducts of 0.5 day pseudo-pregnant females. Timed pseudo-pregnant females were obtained by mating C57Bl/6J x CBA/J F1 females to vasectomized C57Bl/6J x CBA/J F1 males and checking for vaginal plugs. Approximately twenty two-cell embryos were implanted per mouse.

#### *MEMS nanoinjection devices*

Nanoinjector design, fabrication and release were performed as described previously (Aten et al 2012). The release process was followed by thorough rinses in sterile deionized water and isopropanol. The chip was then in a clean and sterile condition. The released chip was adhesively bonded to the inner side of a 35 mm cellculture dish lid. The lid served as the dish for submerging the chip in phosphate buffered saline (PBS) during nanoinjection.

#### *Nanoinjection, IEN, and microinjection*

For the all comparative studies, harvested zygotes were pooled and made available to the microinjection technician and the nanoinjection/IEN technician. All transfer surgeries of two-cell stage embryos into surrogate females occurred the day following injections. Viability was recorded at 24 hours after injection and after births see figure 5.1.

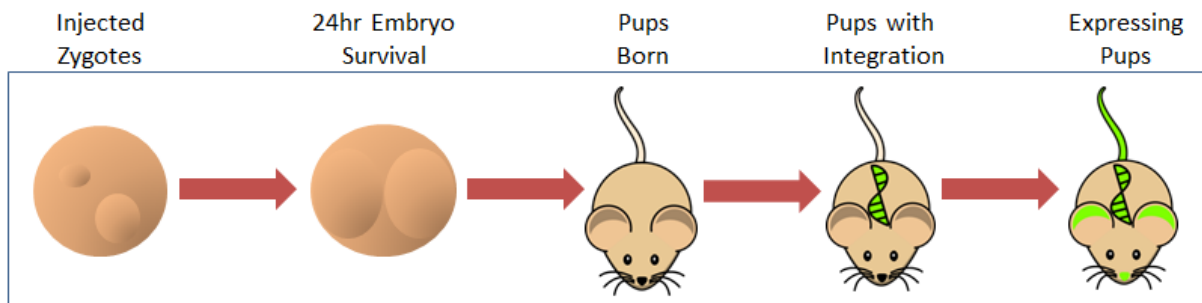


Figure 5.1 Sequence of survival, integration, and expression analysis in large scale comparison studies.

Nanoinjection and IEN were performed in PBS at room temperature and zygotes remained on the MEMS chip for less than 30 minutes. The nanoinjection lance held (+)1.5 volts while 0.25  $\mu\text{l}$  of  $\sim 15 \text{ ng}/\mu\text{l}$  DNA solution was dispensed over the lance from a holding pipette using a syringe pump. The (+) voltage was maintained to allow DNA accumulation for 30-90 seconds. The (+) voltage was also maintained while positioning the lance in the embryo. Nanoinjection employed a repulsion voltage of (-)1.5 volts for 10 seconds. IEN's repulsion

patterns were either #1- 10 0.5 millisecond pulses 2 volts above decomposition, or #2 2 sets of 7 0.5 millisecond pulses 2 volts above decomposition. The lance was then withdrawn from the zygote

Microinjection was performed in M2 under oil at room temperature. Two picoliters of a 2-3 ng/ $\mu$ l DNA solution was microinjected using an Eppendorf Femtojet or an Eppendorf CellTram microinjection system until there was obvious slight swelling of the pronucleus.

#### *Genotypic and phenotypic testing*

DNA was purified using Qiagen DNeasy tissue kit or through digestion with proteinase K and washes with isopropanol. Each sample was subjected to PCR for transgene as well as for mouse  $\beta$ -actin. Mouse  $\beta$ -actin primers (forward 5'-GTGGGCCGCTCTAGGCACCA-3' and reverse 5'-CGGTTGGCCTTAGGGTTCAGGG-3') to yield a 244 bp product. EGFP primers (forward 5'-ATGGTGAGCAAGGGCGAGGA-3' and reverse 5'-TTGTACAGCTCGTCCATCCG -3') to yield a 716 bp product. YAC specific primers Ade (Forward 5'-GGCCTCACAACTCTGGACAT-3' and reverse 5'-ACCATACCTGGCAAGTGAGC-3'), Trp1 (forward 5'-GCCCAATAGAAAGAGAACAATTGACC-3'), and Leu2 ( forward 5'-ATTGATTGATTCTGCCGCCATGATCC-3' and reverse 5'-CAAGATAGTGGCGATAGGGTTGACC'3').

Flow cytometry was performed on samples of thigh muscle, brain, spleen, and gut to detect EGFP expression. Samples were homogenized in 2 ml of Hanks, and passed through a 70  $\mu$ m filter. Readings were obtained with a BD Biosciences FACSCanto cytometer were analyzed using Diva software (BD Biosciences) and Summit software (Dako-Cytomation).

#### *DNA sequencing and southern blot*

EGFP PCR products from representative mice were submitted to the BYU DNA Sequencing Center for Big Dye sequencing. DNA samples from PCR positive pups and WT controls were submitted to TransViragen (Research Triangle Park, NC) for southern blot analysis. For southern blotting, genomic DNA samples were digested with PstI and blots were hybridized with a 716 bp chemiluminescent probe (forward primer 5'-ATGGTGAGCAAGGGCGAGGA-3', reverse primer 5'-TTGTACAGCTCGTCCATCCG-3').

*Statistical analysis of survival, integration, and expression data*

The viability, birth, integration, and expression data were analyzed using statistical methods for the two-tailed Fisher's exact test for 2x2 contingency tables (Everitt 1992; Upton 1992) using the statistical software package SAS (S.A.S Institute). Confidence intervals for binomial proportions were computed using Jefferys 95% confidence interval (Brown et al. 2001). The Jeffreys confidence intervals were calculated with a confidence level of  $(1-\alpha)$  for  $x$  successful events (births, expressing pups, etc.) out of  $n$  attempts (injections performed, pups observed, etc.) with  $\alpha = 0.05$ .

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