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ORIGINAL ARTICLE

SLIM microscopy allows for visualization of DNA-containing liposomes designed for sperm-mediated gene transfer in cattle

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Abstract

Naked DNA has been shown to bind naturally to the sperm, a method called sperm-mediated gene transfer (SMGT). Based on these observations, we examined the efficiency of exogenous DNA binding to sperm using liposomes. In this experiment, we analyzed methods to select frozen-thawed bovine sperm, and evaluated the binding of exogenous DNA to those sperm. To determine the optimal selection method, we used Computer-Assisted Sperm Analysis (CASA). Percoll or Swim-Up were used to select sperm, followed by incubation up to 3 h with the liposome-DNA complexes. The samples were collected after 1 h and after 3 h. We used enhanced green fluorescent protein (eGFP) in combination with the liposomes as a marker for exogenous DNA binding. Five treatments per selection method were analyzed: (1) no incubation, no liposomes and no DNA, (2) incubation with no liposomes and no DNA, (3) incubation with liposomes and no DNA, (4) incubation with liposomes and 1 µg of DNA and (5) incubation with liposomes and 10 µg of DNA. The CASA results for total motility and rapid motility were statistically significant ($P < 0.01$) between the control and the other treatments in the Percoll group as opposed to Swim-Up. Swim-Up was therefore chosen as the optimal selection method. In order to determine if the liposome-DNA complex had bound to sperm, real time PCR was used to detect GFP DNA and images of the sperm were analyzed using the Spatial Light Interference Microscopy (SLIM). SLIM confirmed the presence of liposomes on the sperm head and tail.

Keyword Liposome · Sperm · GFP · SLIM · Microscopy · Sperm-mediated gene transfer

Introduction

Declining poverty and increases in the middle class, especially in China, have led to an increase in consumption of meat and dairy products [17]. By 2050, the population is expected to increase to ~9 billion people and much of that growth will occur in developing countries. One way to meet the food demand is by genetically modifying animals. Pronuclear DNA injection has long been the most used protocol to introduce new genetic material into the genome. Pronuclear injection requires specialized equipment and highly trained staff. In the last 30 years, research has focused on different methods to produce transgenic animals, including sperm-mediated gene transfer (SMGT), which requires less skill [30]. Sperm-mediated gene transfer has been successfully used to transfer DNA to embryos using the sperm's ability to bind naked DNA [8, 12, 29]. In this case, sperm is used as the vector for specific gene transfer, meaning exogenous DNA is transported into the oocyte during fertilization. Successful SMGT has been reported in mice [2, 6, 12, 15], cows [20, 27, 29], and other species.

Augmentation techniques are also used with SMGT. These include methods that use electroporation or liposomes to drive the sperm to take up the transgene DNA. Liposome/DNA delivery methods are another technique under study for introducing exogenous DNA into cells and embryos. Liposomes are small structures consisting of membrane-like

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lipid layers or bilayers, which can actually protect foreign DNA from digestion by proteases and DNAse [26]. Cationic liposomes are capable of spontaneously interacting with DNA molecules, giving rise to lipid-DNA complexes [5]. It is proposed that ionic interactions between the positively charged hydrophilic exterior of liposomes and the negatively charged phosphate groups of DNA molecules are responsible for the lipid-DNA complex formation [16]. Delivery of foreign DNA into the target cell occurs via fusion of the lipid-DNA complex with the cell membranes. Under appropriate conditions, exogenous DNA can be transferred into cells and a portion of this DNA becomes localized in the nucleus [5].

An advantage of cationic liposomes is that DNA size does not seem to be a limiting factor during transfection [4, 10]. Liposome-mediated gene transfer has been shown to be a highly efficient method for transforming cultured cells with foreign DNA. Liposomes have also been a very effective tool to transfect spermatozoa [19]. The major advantage to sperm-mediated transfer for producing transgenic animals is its simplicity. However, the disadvantage of this technique is the decreased ability of the host's genome to incorporate DNA presented and the instability of replicating previous experiments.

In a previous study cationic lipids combined with neutral lipids were used to enhance binding of DNA to sperm. Although, the results were very poor and to the best of our knowledge, in a representative study, just 3.6% of blastocysts were transfected [7].

In this work, we aim to shed light on the disparity in transfection outcomes between cells and sperm. To this end we set out to: (1) determine the best method for the sperm selection (we compared the most common protocols: Discontinuous Gradients (Percoll) and Swim-Up), (2) investigate co-incubation time (sperm and liposome), and (3) compare DNA concentration to bind (1 or 10 micrograms) using three different experimental methods: (1) computer-assisted sperm analysis (CASA), (2) quantitative polymerase chain reaction (qPCR), and (3) quantitative phase imaging [21] to characterize the attachment of liposomes.

Materials and methods

Experimental design

For these experiments three different bulls were used, all were previously tested for in vitro embryo production. Six straws of frozen semen per replicate were thawed (two for each bull). We did not have access to fresh bull semen for this experiment. All straws were collected into the same tube and then divided. Two different sperm selection methods were used, Swim-Up and Percoll gradient. For each method, zero, one, or ten micrograms of DNA were added to liposomes. Sperm motility with no liposome and no DNA after 1 and 3 h of incubation was measured as a control. The liposomes were then incubated with DNA and combined with sperm to be incubated for 1–3 h. After the semen was processed, the samples were analyzed with CASA (Hamilton Thorne Bioscience, Beverly, MA) then they were divided into sub-groups: Percoll Liposome No DNA, Percoll Liposome 1 µg, Percoll Liposome 10 µg, Swim-Up Liposome No DNA, Swim-Up Liposome 1 µg, Swim-Up Liposome 10 µg; these groups were performed in 1-h and 3-h incubation periods for a total of 12 sub-groups. At each time point, three samples for each experimental group were collected: CASA, qPCR, and a sample for imaging. Samples for PCR analysis were placed in a − 80 °C freezer for DNA extraction. A 10 µl drop of each sample was put on a slide for quantitative phase imaging [21], giving us the opportunity to evaluate the percentage of sperm with liposomes attached as well as characterize the dry-mass of these attached structures [22]. The experiment was replicated six times.

Sperm selection methods

Semen straws were removed from the liquid nitrogen tank and thawed to 37 °C for 40 s. After thawing, the semen was processed with two standard protocols: Swim-Up or Percoll (45–80%). Discontinuous gradients were prepared by combining Sperm-TALP (Tyrode's Albumin Lactate Pyruvate) and ISO-Percoll. Sperm-TALP-basic-medium [25], was supplemented with pyruvic acid (Sigma-Aldrich, St. Louis, MO) and gentamycin sulfate (Sigma-Aldrich, St. Louis, MO). ISO-Percoll was made by combining sodium bicarbonate with Earle's Balanced Salt Solution (EBSS) (Thermo Fisher Scientific, Waltham, MA) and vortexed in a tube, then combined with Percoll® (Sigma-Aldrich, St. Louis, MO). The pH was adjusted to 7.4. ISO-Percoll and Sperm-TALP were combined to create 45% and 80% mixture of gradients (45% on the top of 90%).

Semen was slowly pipetted down the side of the tube to create a third layer on top of the 45% gradient and then centrifuged at 460 × g for 25 min. The supernatant was discarded and the pellet was washed in one milliliter of Sperm-TALP and centrifuged at 250 × g for 10 min. The supernatant was again discarded and the pellet was suspended in one milliliter of Sperm-TALP and centrifuged at 170 × g for 10 min. The supernatant was removed and 50 ⌈l of Sperm-TALP was added to the pellet.

Swim-Up

The sample of thawed semen was layered carefully under 1 ml of equilibrated sperm-TALP medium with 6 mg of BSA (Bovine Serum Albumin) per ml in a centrifuge tube. After

loading, the tube was placed in an incubator at 39 °C for 1 h. After incubation, 400 µL of the upper fraction (containing the selected sperm) was collected, placed in a tube, and centrifuged for 10 min at 160 × g [28].

DNA constructs

pIRES2-EGFP is an IRES-containing bicistronic vector for expressing a gene of interest together with EGFP(http://www.dmlim.net/vectors/pIRES2-EGFP/pIRES2-EGFP-map.pdf). The EGFP is driven by the CMV promoter and the neomycin/kanamycin resistance cassette are driven by the SV40 early promoter. (Fig. 1). The qPCR primers used to detect pIRES2-EGFP (Clonetech Laboratories Inc. (now Takara Bio USA Inc.) were (CATGGTCCTGCTGGAGTT CGTG) and (CGTCGCCGTCCAGCTCGACCAG).

Liposome preparation

The cationic lipid, 3-(trimethyl ammonium iodide) 1,2 dimystryl-propanediate (TAID) was synthesized by Russell [24] and used for the subsequent studies. The neutral lipid, L-α Dioleoyl phosphatidyl-ethanolamine (DOPE) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and used without further purification. DOPE was reconstituted in a 9:1 ration of $CHCl_3$:MeOH. Once mixed, the TAID and DOPE were dissolved in the organic solvent chloroform ($CHCl_3$; Sigma Aldrich, St. Louis, MO) at a molar ratio of 2:1 in a round bottom flask. A rotary evaporator was then used to concentrate the lipid at the bottom of the flask at 40–50 °C. When the chloroform was completely evaporated, 3 µl of TALP were added to the round bottom flask containing the lipid film. A cell scraper was then used to scrape the layer of lipids from the glass surface. The sample was then pipetted up and down for 1 min followed by 1 min of vortexing. The flask was placed in an incubator at 37 °C for 15 min in a 5% CO_2 in air atmosphere. 50 µl of the liposome-TALP mix were added to tubes containing 0, 1, or 10 micrograms of DNA. Liposome/DNA complexes were incubated at 37 °C for 15 min at 5% CO_2. Collected sperm was added to medium alone (control) or medium containing liposome/DNA complexes. The 50 µl were then added to 150 µl of TALP and sperm. The final concentration of the solution is 150 µM lipids, 1 or 10 µg DNA, and sperm in TALP.

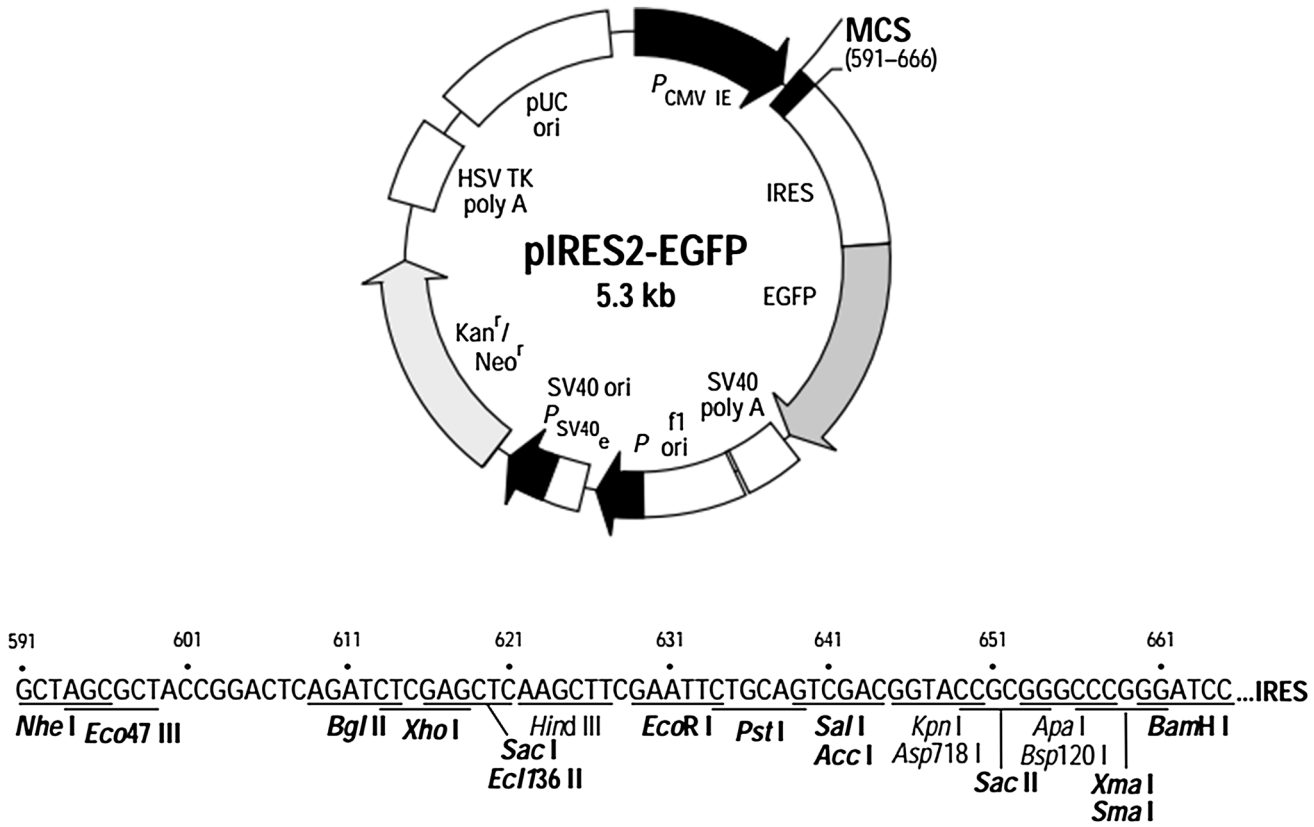

Fig. 1 pIRES2-EGFP vector construct. pIRES2-EGFP is an IRES-containing bicistronic vector for expressing a gene of interest together with EGFP. The EGFP is driven by the CMV promoter and the neomycin/kanamycin resistance cassette is driven by the SV40 early promoter. BD Biosciences Clontech: http://www.bdbiosciences.com Protocol # PT3267-5 2 Version # PR29951 (http://www.dmlim.net/vectors/pIRES2-EGFP/pIRES2-EGFP-map.pdf)

Computer-assisted semen analysis (CASA)

A CASA IVOS system was used to measure total motility, rapid motility, and progressive motility of sperm. The software was set per the manufacturer's recommendations for the assessment of motility characteristics of bovine bull spermatozoa as follows: frames acquired: 30; frame rate: 60 Hz/s; minimum contrast for cell detection: 80; minimum cell size: 5 pix; Progressive VAP 50 μ/s; straightness threshold: 70%; slow VAP cut off: 30 μ/s; slow VSL cutoff: 15 μ/s; and magnification factor 1.92. For each sample, 3 μl was removed and 10 microscopic fields were analyzed. The image knob was adjusted until the sperm were clearly visible on the monitor.

Sperm genomic DNA extraction

Frozen samples at − 80 °C were thawed to room temperature. Samples were centrifuged at 15,600 × g for 5 min and the supernatant was removed. 500 μl of 70% ethanol were added to the pellet and centrifuged again at 15,600 × g for 5 min. Cells were lysed by adding 500 μl of lysis buffer, which was composed of 2 ml of 5 M NaCl, 1 ml of 1M tris, 2.5 ml of 1M EDTA (Thermo Fisher Scientific, Waltham, MA), 5 ml 10% SDS, and 89.5 ml ddH_2O for a final volume of 100 ml. Then 2.5 μl of 0.5% Triton® X-100, 21 μl of 1M dithiothreitol (DTT), and 40 μl of 10 mg/μl of proteinase K (Invitrogen- Thermo Fisher Scientific, Waltham, MA) were added to the sample. Samples were then vortexed and incubated at 50 °C overnight on a shaker with moderate shaking. The following day the samples were centrifuged for 10 min at 15,600 × g and the supernatant was transferred to a new 1.5 ml tube. In order to precipitate the DNA, 1 μl of 20 mg/ml glycogen and 1/10 volume of 3M NaAc was added to the supernatant. Then 2 volumes of ice-cold absolute ethanol were added and placed in − 80 °C for 1–2 h. The DNA was then pelleted by centrifuging the sample for 20 min at 15,600 × g. The supernatant was removed and the pellet was then washed with 500 μl of 75% ethanol and centrifuged for 10 min at 15,600 × g. Ethanol was pipetted slowly from the tube and then the tube was left at room temperature until the remaining ethanol had evaporated. The DNA was dissolved in 30 μl of TE buffer. The TE buffer was composed of 10 ml of 1 M TRIS pH 8.0, 1 ml of 1M EDTA, and brought to a final volume of 1000 mL with ddH_2O. The DNA was left at 4 °C overnight and the concentration was measured the next day using a NanoDrop 1000 Spectrophotometer nucleic acid analyzer (Thermo Fisher Scientific, Waltham, MA). The DNA samples were stored at − 20 °C.

Quantitative real-time PCR (qRT-PCR) analysis

qPCR was performed using SYBR→ Select Master Mix for CFX (Thermo Fisher Scientific, Waltham, MA). A total volume of 10 μl contained 5 μl of SYBR Select Master Mix, 0.4 μl of forward and reverse primer, 4 μl of gDNA and 0.6 μl of ddH2O. The thermocycling profile was the following; stage 1: 50 °C for 2 min, stage 2: 95 °C for 10 min, stage 3: 95 °C for 15 s then 65 °C for 1 min repeated for 40 cycles, stage 4: 95 °C for 15 s then 60 °C for 15 s. Three replicates were carried out for quantification of the target gene. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was amplified for each sample as a housekeeping gene for bovine sperm and GFP was used as a primer to amplify GFP plasmid. Primers to detect eGFP [(pIRES2-EGFP, Clonetech (now TaKaRa)] were (CATGGTCCTGCTGGAGTTCGTG) and (CGTCGCCGTCCAGCTCGACCAG). The control primers for YWHAZ used were forward primer (GCATCCCAC AGACTATTTCC) and reverse primer (GCAAAGACAATG ACAGACCA).

SLIM analysis

Ten μl of sperm was added to a glass slide and the sample was drawn across the slide to spread it evenly. These slides were air-dried and stored at 4 °C until ready for analysis. Images were taken of the slides using the SLIM instrument described in [14]. QPI techniques yield a phase rather than the intensity map, which allows for quantitative measurements on transparent specimens, such as unlabelled cells. In reproductive research, such systems have found fertile ground in applications characterizing whole embryos [18, 31] as well as sperm [3, 13]. However, the constraints on the image quality necessary to characterize liposomes are very stringent, i.e., the the sensitivity of the QPI method must be very high. To meet this challenge, in this work we choose spatial light interference microscopy (SLIM), which is highly sensitive in both space in time, due to, repsectively, the use of white light, which avergaes the speckles, and common path interferometric geometry, which insures phase stability [9]. SLIM has been used recently to characterized the topography and refractometry of sperm [14].

SLIM was used to detect the presence and size of attached liposomes on the sperm head and tail. To measure the diameter of the liposome, profilometry of the liposomes was performed using ImageJ software (NIH, Bethesda, MD).

Statistical analysis

The results for CASA were analyzed as the mean rapid, progressive, or total motility for 5 different groups in 5 replicates. The statistical differences between these were

analyzed, in Excel, using a Chi square test with Yates correction. Differences were considered to be significant for $P < 0.05$. A Chi square test with Yates correction was also used to evaluate qPCR data. There was no significant difference between 1 h and 3 h of incubation based on CASA results, using generalized least squares (GLS) ANOVA analysis. Therefore, the average percent motility of the different groups for 1 h and 3 h were combined to determine the differences between the methods used. These were also analyzed using GLS ANOVA (SPSS version 19. IBM, New York) to evaluate the differences between groups based on the percent of liposomes attached to sperm using post hoc analysis with Bonferroni correction.

Results

Sperm incubation with plasmid GFP

Sperm motility was measured to determine the optimal method to attach DNA to sperm for SMGT. The sperm was analyzed using CASA to measure total motility, rapid motility, and progressive motility of sperm. The average total motility, progressive motility, and rapid motility of sperm using Percoll and Swim-Up selection after 1 h are shown in Table 1, and after 3 h in Table 2. There was no significant difference when we evaluated the time variable using generalized least squares (GLS) ANOVA. Therefore, the average percent motility of the different groups for 1 h and 3 h were combined to determine the differences between the methods used. These were also analyzed using GLS ANOVA (Table 3). The percentage of total motility (TM), progressive motility (PM), and rapid motility (RM) for Percoll were significantly decreased in sperm with liposomes compared to the control ($P < 0.05$). When we analyzed the Swim-Up groups we found a statistical difference between the control group and the group containing 10 µg of DNA but not when we compared the control with 1 µg of DNA, for the parameter TM, PM, and RM (Table 3). Furthermore the Swim-Up group with 1 µg of DNA was no statistically different with the two control (Swim-Up and Percoll), while the Percoll 1 µg of DNA

Table 1 CASA results: The average motility (±SE) for each group with Percoll and Swim-Up selection after 1 h

Method	Experiment	Total motility Average	Progressive motility	Rapid motility
Percoll	Control (T0)	87.50 (2.67)	73 (0)	82.30 (2.67)
	No liposome	41.50 (26.75)	26.50 (19.94)	30.00 (20.51)
	Liposome no DNA	26.25 (3.20)	14.75 (2.50)	18.00 (3.56)
	Liposome 1 µg DNA	38.00 (17.03)	20.25 (9.07)	25.25 (12.58)
	Liposome 10 µg DNA	24.50 (4.43)	13.50 (2.65)	15.00 (3.37)
Swim-Up	Control (T0)	61.00 (31.10)	44.75 (20.88)	51.50 (25.077)
	No liposome	77.25 (9.18)	53.00 (7.26)	59.50 (3.87)
	Liposome no DNA	68.50 (11.70)	49.50 (11.70)	51.50 (10.41)
	Liposome 1 µg DNA	60.75 (31.32)	49.00 (26.39)	54.00 (30.11)
	Liposome 10 µg DNA	56.75 (17.29)	33.50 (14.84)	37.75 (14.50)

The first control (T0) is the motility of the sperm immediately after selection

Table 2 CASA results: the average motility (±SE) for each group with Percoll and Swim-Up selection after 3 h

Method	Experiment	Average		
		Total motility	Progressive motility	Rapid motility
Percoll	Control (T0)	87.50 (2.67)	73 (0)	82.30 (2.67)
	No liposome	56.50 (7.50)	43.00 (0)	49.00 (3.46)
	Liposome no DNA	60.00 (8.08)	46.50 (6.35)	52.50 (5.20)
	Liposome 1 µg DNA	62.50 (14.43)	18.00 (9.24)	55.00 (17.32)
	Liposome 10 µg DNA	29.00 (9.24)	52.00 (20.78)	23.00 (10.39)
Swim-Up	Control (T0)	61.00 (31.10)	44.75 (20.88)	51.50 (25.077)
	No liposome	69.50 (4.04)	48.50 (16.74)	48.50 (16.74)
	Liposome no DNA	44.00 (42.72)	30.00 (30.02)	31.50 (28.29)
	Liposome 1 µg DNA	59.50 (10.97)	41.50 (9.81)	47.00 (10.39)
	Liposome 10 µg DNA	34.00 (28.87)	31.00 (25.40)	31.00 (25.40)

The first control (T0) is the motility of the sperm immediately after selection

was statically different compare the Percoll control but not with the Swim-Up control.

To determine if plasmid DNA had attached to the sperm, genomic DNA was extracted and then analyzed by qPCR. Five sets of triplicates were quantified for each group, resulting in a total of 15 samples. A number one was given to each positive Ct value for the primers, the one values were added together for each treatment and then eGFP was divided by YWHAZ to show what percent of DNA had bound to sperm. For samples where no eGFP DNA was added, there were no positive Ct values. 66.67% of sperm selected using Percoll and incubated with liposomes 1 µg of DNA had positive Ct values, 100% with 10 µg of DNA had positive Ct values. Sperm selected using Swim-Up had 57.14% of eGFP DNA attach with 1 µg of DNA and 100% with 10 µg of DNA (Table 4). These values were compared using a Chi square test with Yates correction. No difference was found between the Swim-Up and Percoll selection methods when the same amount of DNA was added. Differences were seen in the attachment of DNA between groups that had 0, 1, or 10 µg of DNA added (Table 5).

Liposome verification with SLIM microscope

After the sperm had been incubated for 1 or 3 h, a drop of the liposome-sperm complex was placed on a glass slide and then air-dried to be evaluated using SLIM (Table 6). The average size of a liposome can range from small (0.025 µm) to large (2.5 µm) sized vesicles [1]. The size of liposomes did not vary significantly between groups and the average size was as expected (~ 1.9 µm) (Table 6). SPSS version 19 (IBM, New York) was used to evaluate the differences between groups based on the percent of liposomes attached to sperm using post hoc analysis with Bonferroni correction. There was a significant difference between time point 0 and 1 h, and time 0 and 3 h but not between 1 and 3 h. This

Table 3 CASA results without time comparison: comparison of CASA results for total motility, progressive motility and rapid motility with standard deviation

	Total motility	Progressive motility	Rapid motility
Percoll control	68.25 (24.14)^{A}	53.87 (22.49)^{A}	61 (25.12)^{A}
Percoll Liposome No DNA	43.12 (18.92)^{BC}	30.62 (17.56)^{BC}	35.25 (18.90)^{BC}
Percoll Liposome 1 ug DNA	50.25 (19.62)^{AB}	36.12 (22.55)^{B}	40.12 (21.2)^{B}
Percoll liposome 10 ug DNA	26.75 (7.13)^{C}	15.75 (6.73)^{C}	19 (8.33)^{BC}
SwimUp control	67.19 (22.81)^{A}	47.75 (16.81)^{AB}	52.75 (19.24)^{AB}
SwimUp Liposome No DNA	56.25 (31.82)^{ABC}	39.75 (23.53)^{B}	41.5 (22.44)^{BC}
SwimUp Liposome 1 ug DNA	60.12 (21.73)^{AB}	45.25 (18.87)^{AB}	50.5 (21.19)^{AB}
SwimUp Liposome 10 ug DNA	45.37 (25.16)^{BC}	32.25 (19.31)^{BC}	34.37 (19.49)^{BC}

The average motility of sperm that was incubated for 1 and 3 h was combined to exclude the timing factor

^{ABC}Least square means (± SE) within each row with different superscripts differ ($P < 0.05$)

Table 4 qPCR results: qPCR products that had positive Ct values for pEGFP and control gene YWHAZ

Gene	P0	P1	P10	S0	S1	S10
GFP/YWHAZ %	0	66.67	100	0	57.14	100

Methods used to select sperm were *P* Percoll and *S* Swim-Up. The DNA concentration added to the sperm were 0, 1 and 10 µg

P0 Percoll 0, *P1* Percoll 1, *P10* Percoll 10, *S0* Swim-Up 0, *S1* Swim-Up 1, *S10* Swim-Up 10

Table 5 Comparison of qPCR data: the comparison is based on the percentage sperm with GFP divided by the percentage of sperm with the control gene YWHAZ

	P0	P1	P10	S0	S1	S10
P0	Not sig	1%	1%	Not sig	1%	1%
P1	1%	Not sig	1%	1%	Not sig	1%
P10	1%	1%	Not sig	1%	1%	Not sig
S0	Not sig	1%	1%	Not sig	1%	1%
S1	1%	Not sig	1%	1%	Not sig	1%
S10	1%	1%	Not sig	1%	1%	Not sig

The resulting values are given as a percentage in the table. For each group, the percentage was compared using Chi square analysis

P0 Percoll 0, *P1* Percoll 1, *P10* Percoll 10, *S0* Swim-Up 0, *S1* Swim-Up 1, *S10* Swim-Up 10. $P > 0.01$

Table 6 Average size of liposomes: The average liposome size in µm (± SE) found on sperm head, middle piece and tail using SLIM imaging

	1 h			3 h		
	Liposome NoDNA	1 µg	10 µg	Liposome NoDNA	1 µg	10 µg
Swim-Up	1.74 (0.67)	1.82 (0.58)	1.84 (0.64)	2.35 (1.17)	2.12 (0.89)	1.92 (0.64)
Percoll	1.69 (0.49)	2.11 (0.72)	1.83 (0.63)	1.6 (0.71)	2.03 (0.81)	2.2 (0.66)

Table 7 Percent of liposomes bound to sperm: table showing the average number of liposomes (± SE) bound to sperm for each group at time 1, and 3

Time	Group	Average
1	Percoll control	1.80 (0.87)
	Percoll Liposome 10 µg	6.51 (1.96)
	Percoll Liposome 1µ g	5.85 (3.12)
	Percoll Liposome No DNA	10.59 (5.57)
	Swim-Up control	2.85 (0.83)
	Swim-Up Liposome 10 µg	10.74 (1.43)
	Swim-Up Liposome 1 ug	10.26 (3.80)
	Swim-Up Liposome No DNA	8.46 (3.70)
3	Percoll control	0.95 (0.80)
	Percoll Liposome 10 µg	7.04 (6.37)
	Percoll Liposome 1 µg	7.75 (3.36)
	Percoll Liposome No DNA	5.18 (2.22)
	Swim-Up control	3.42 (1.21)
	Swim-Up Liposome 10 µg	12.06 (2.14)
	Swim-Up Liposome µg	3.52 (3.05)
	Swim-Up Liposome No DNA	1.80 (2.55)

was determined by eliminating groups and evaluating time only to determine if the microscope can detect liposomes. The average percentage of liposomes bound to sperm varied between groups (Table 7). This variation was also analyzed using Bonferroni correction. After 1 h of incubation, we found a diference between control and all the other groups except for the 1 µg of DNA group where no difference was found between the control. Similar, results were found in the Percoll and Swim-Up groups. After 3 h of incubation, the percentage of liposomes bound was different between the Percoll control and the other Percoll groups with liposomes. In contrast, when we evaluated the Swim-Up groups we found that only the 10 µg of DNA group was different from the control.

Discussion

Sperm-mediated gene transfer was one of the first methods used to create transgenic animals. Since then microinjection has become one of the most commonly used methods, but it requires greater skill and there are fewer reports of it being used in livestock species. Nuclear transfer is more commonly reported in livestock but also requires more time and skill than SMGT. Although SMGT has been shown to work in a variety of species, there have been a small number of publications that successfully used this method in cattle. For this experiment, we used SMGT in combination with liposomes to deliver plasmid DNA containing a GFP selection marker. We were able to detect that plasmid DNA did bind to the sperm and determined the selection method that least affected sperm motility.

In our experiment, we used cryopreserved bovine bull semen. Sperm were selected with Percoll or Swim-Up before incubation with the liposome construct and DNA; these methods were used to select viable sperm. Our samples were evaluated with different methods: impact on the motility after the liposome co-incubation and presence of the liposome by two methods: qPCR and label-free quantitative microscopy. The motility of sperm was analyzed using CASA to determine the effects of liposomes and the different selection methods on the motility of the sperm. Based on the CASA results that looked at total, progressive, and rapid motility, Swim-Up was the method determined to least affect sperm motility after co-incubation with liposomes and 1 µg of DNA. To determine if plasmid DNA had successfully bound to the sperm, DNA was extracted from sperm and quantified using qPCR. Quantitative phase imaging was used to confirm the presence of liposomes on the individual sperm and qPCR detected plasmid GFP DNA. With SLIM imaging, we were able to see for the first time, to the best of our knowledge, where liposomes attach to sperm. We saw that liposome distribution is random and equally distributed between head and tail. This information could help explain the low yields in the DNA integration with the embryo DNA obtained from other research groups [7].

This is the first report using quantitative phase imaging to measure liposomes and visualize their attachment to the sperm head, middle piece, and tail. When we evaluated the percentage of sperm with liposomes, we found only a small fraction of sperm had liposomes attached (Fig. 2). Further, in all sperm with liposomes on the head we did not find the acrosome, we could speculate that the liposome attachment induced the acrosome reaction (Fig. 2b, c) or that the liposome only bound to acrosome reacted sperm. This hypothesis is supported by several authors who have shown a good presence of exogenous DNA but only a small number of embryos had the DNA integrated. For example one study saw that 22% of blastocysts showed

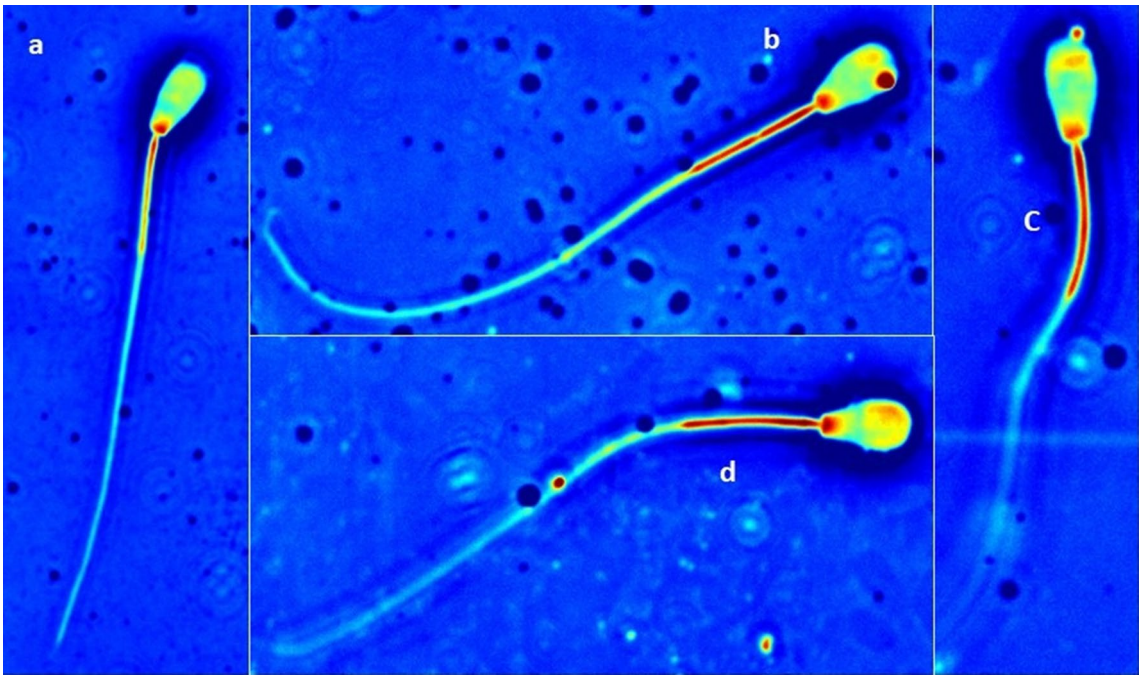

Fig. 2 SLIM images. Images were obtained using jet color map on imageJ with SLIM microscope. Liposomes appeared as red spots on the head and tail because those regions had a higher density. **a** Spermatozoa lacking acrosome, no liposome attached; **b** and **c** Spermatozoa lacking acrosomes but liposome attached to sperm head; **d** completely intact spermatozoa with acrosome and liposome attached to tail

the presence of the desired plasmid DNA using PCR, in this case the blastocysts were made by incubating plasmid DNA with sperm for 2 h and then performing IVF. In this experiment, the authors analyzed 976 blastocysts, but the presence of the transgene could not be verified as proof of integration because the construction used was plasmid DNA and could not integrated into the blastocysts [29]. Another study used AI (artificial insemination) instead of IVF; they inseminated 210 heifers, 41 of which gave birth and but only 1 tested positive for the inserted DNA (3.3%) [27]. A higher percent of edited blastocysts were seen when electroporation was applied to sperm. While 3.5% of embryos showed homologous recombination when sperm was incubated with DNA and no electroporation was applied, an increase to 55% of embryos had homologous recombination when electroporation was applied [23]. Reagents such as FuGENE® 6 have also been used to insert DNA into embryos by incubating FuGENE® 6 with sperm for SMGT, but only 3.6% of blastocysts expressed GFP [7]. Most of these studies had a blastocyst rate of at least 20% and examined between 30 and 100 blastocysts for each group. It is appropriate to mention that all these negative results are in the bovine species while pigs have a much higher success rate, 50–60% of piglets born contain the transgene [11].

Conclusions

In conclusion, we have shown that Swim-Up is the preferred method to select sperm for SMGT and it is possible co-incubate sperm and liposomes for up to 3 h without adverse effects on sperm motility. Moreover, we showed that the SLIM microscopy system is able to identify liposomes bound to sperm without any kind of dye or marker. Further studies will be required to produce high numbers of transgene transformed bovine blastocysts with SMGT based on the results obtained in this study.

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Compliance with ethical standards

Conflict of interest The authors (M.R., S.N.L., M.E.K. and M.B.W.) declare that they have no conflict of interest. G.P. has financial interest in Phi Optics, Inc., a company developing quantitative phase imaging technology for materials and life science applications.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. We did not used any live animals for these studies. The frozen semen used was from a commercial semen vendor. We purchased the semen used it these studies and had no contact with the bulls used for the in vitro

fertilization. Our University does not require IACUC (animal care and use) protocols when using purchased tissues or abattoir materials. We therefore see no ethics issues with the present study.

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