

Analysis of sperm motility using optical tweezers

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1 Introduction

In fertility physiology studies, the speed of progression (SOP) score is often used as a key parameter in the determination of overall motility score of a semen sample where motility score = (% motile) * (SOP score of sample).^{1,2} The motility score is used to estimate the probability of a successful fertilization. The SOP score takes on discrete values from 1–5, where 5 qualitatively represents the fastest swimming sperm and 1 represents the sperm that exhibit the least amount of forward progression.¹ Since the SOP score is qualitative, it may be subject to variation between individuals. Swimming velocity, trajectory curvature, displacement, and lateral head movement are other parameters used to assess sperm motility.

Optical forces from a single beam gradient laser trap can be used to confine and manipulate microscopic particles.^{2,3} These optical traps have been used to study laser–sperm interactions and sperm motility by measuring sperm swimming forces.^{4–9} These studies determined that the minimum amount of laser power needed to hold the sperm in the trap (or the threshold escape power) is directly proportional to the sperm's swimming force ($F = Q * P / c$, where F is the swimming force,

Abstract. This study examines the use of optical trapping as a quantitative measure of sperm motility. The effects of laser trap duration and laser trapping power on sperm motility are described between sperm swimming force, swimming speed, and speed of progression (SOP) score. Sperm (SOP scores of 2–4) were trapped by a continuous-wave 1064 nm single-point gradient laser trap. Trap duration effects were quantified for 15, 10, and 5 seconds at 420 mW laser power. Laser power effects were quantified at powers of 420 mW, 350 mW, 300 mW, and 250 mW for five seconds. Swimming force, swimming speed, and SOP score relationships were examined at a trap duration and trapping power shown to minimally affect sperm motility. Swimming forces were measured by trapping sperm and subsequently decreasing laser power until the sperm escaped the trap. Swimming trajectories were calculated by custom-built software, and SOP scores were assigned by three qualified sperm scoring experts. A ubiquitous class of sperm were identified that swim with relatively high forces that are uncorrelated to swimming speed. It is concluded that sperm swimming forces measured by optical trapping provide new and valuable quantitative information to assess sperm motility. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2337559]

Keywords: optical tweezers; sperm swimming force; sperm swimming speed; speed of progression (SOP) score.

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P is the laser power, c is the speed of light in the medium, and Q is the geometrically determined trapping efficiency parameter⁴). These studies used the measurement of sperm swimming forces to evaluate sperm viability by characterizing the effects of cryopreservation of sperm⁶ and comparing the motility of epididymal sperm to ejaculated sperm.⁵ The human sperm studies found that as swimming speed increased, the average escape power also increased.⁹ This correlation was found for a population of relatively slow-swimming sperm. SOP scores were not assigned to the sperm in this study; however, sperm of these speeds are typically assigned an SOP score of 2.

It is the purpose of the present study more specifically to determine if there is a quantitative relationship between sperm swimming forces, their swimming speeds, and the SOP scores (ranging from 2–4). This study also measures the effects of the laser trap duration and laser trapping power on sperm motility to determine the values at which their effects are statistically negligible. In this paper we describe the use of a laser optical trap to measure the swimming forces of a large number of individual sperm in a semen sample, thus expanding the quantitative parameters available for assessment of sperm motility and semen quality.

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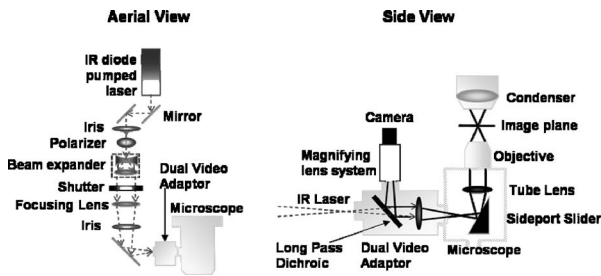


Fig. 1 Optical schematic: Layout of microscope path and optical tweezers.

2 Materials and Methods

2.1 Hardware and Optical Design

A single-point gradient trap was generated using an Nd:YVO₄ continuous-wave 1064 nm wavelength laser (Spectra Physics, Model BL-106C, Mountain View, CA) coupled into a Zeiss Axiovert S100 microscope and a 40 \times , phase III, NA 1.3 oil immersion objective (Zeiss, Thornwood, NY), which is also used for imaging as previously published.¹⁰ The optical design is shown in Fig. 1. Laser light is reflected off two dielectric mirrors to orient the beam parallel to the table and along the optical axis of the microscope. The beam is expanded by two lenses (plano-concave lens, $f = -25.5$ mm at $\lambda = 1064$ nm, and plano-convex lens, $f = 76.2$ mm at $\lambda = 1064$ nm) in order to fill the objective's back aperture. A third lens (biconvex lens, $f = 200$ mm) focuses the beam onto the side port of the dual video adaptor to ensure the beam is collimated at the objective's back aperture.¹¹ The dual video adaptor contains a filter cube with a dichroic that allows laser light entering the side port to be transmitted to the microscope while reflecting visible light to the camera attached to the top port for imaging. A filter (Chroma Technology Corp., Model D535/40M, Rockingham, VT) is placed in the filter cube to block back reflections of IR laser light while allowing visible light to pass. The laser trap remains stationary near the center of the field of view. In order to trap a sperm, the microscope stage is moved to bring the sperm to the laser trap location. The laser trap location is determined prior to each experiment by trapping 10- μ m-diameter polystyrene beads suspended in water within a 35-mm-diameter glass bottom Petri dish. The trap depth within the sample is kept to approximately 5 μ m (approximately one sperm head diameter) above the cover glass. This ensures that the trap geometry is not sensitive to spherical aberrations from the surrounding media. A removable polarizer is used only for power decay experiments (experiment 3) to control laser power. Laser power in the specimen plane is attenuated by rotating the polarizer, which is mounted in a stepper-motor-controlled rotating mount (Newport Corporation, Model PR50PP, Irvine, CA). The mount is controlled by a custom program that allows the experimenter to set the power decay rate (rotation rate of polarizer) and record power decay parameters once the sperm escapes the trap. The specimen is imaged at 30 frames per second by a CCD camera (Sony, Model XC-75, New York, NY), coupled to a variable zoom lens system (0.33–1.6 X magnification) to demagnify the field of view. Analog output (RS-170 format) from the

CCD is wired into the video-in port of a digital camcorder to record sperm swimming for off-line analysis.

2.2 Specimen

Semen samples collected from several dogs were pooled and cryopreserved according to a published protocol.^{12,13} Semen samples were also collected from two dogs, different than the dogs used in the pooled sperm samples, and cryopreserved individually (dog 1: labeled 04-060, dog 2: labeled 05-037). Dog 2's semen sample was subdivided into two samples in order to test for experimental errors in sperm preparation from one day to the next. For each experiment, a sperm sample is thawed in a water bath (37 $^{\circ}$ C) for approximately one to two minutes, and its contents are transferred to an Eppendorf centrifuge tube. The sample is centrifuged at 2000 rpm for 10 minutes (centrifuge tip radius is 8.23 cm). The supernatant is removed and the remaining sperm pellet is resuspended in 1 mL of prewarmed media [1 mg of bovine serum albumin (BSA) per 1 mL of Biggers, Whittens, and Whittingham (BWW), osmolarity of 270–300 mmol/kg water, pH of 7.2–7.4¹⁴]. Final dilutions of 30,000 sperm per mL of media are used in the experiments. The specimen is loaded into a rose chamber and mounted into a microscope stage holder.¹⁵ The sample is kept at room temperature. To quantify the effects of temperature, control experiments were conducted using an air curtain incubator (NEVTEK, ASI 400 Air Stream Incubator, Burnsville, VA) to achieve 37 $^{\circ}$ C at the specimen.

2.3 Experiment 1—Trap Duration

The goal of this experiment is to determine if the duration a sperm is exposed to the laser trap has a significant effect on sperm motility. The effects are tested on sperm from two dog sperm samples pooled together. Laser trapping power is held constant at 420 mW in the specimen plane for durations of 15, 10, and 5 seconds. (Laser power is measured using a photodiode just after the oil immersed objective to define the power in the focal plane.) Curvilinear velocity (VCL, μ m/sec) is measured prior to and post trapping for three to five seconds. VCL is the average velocity of the point-to-point trajectory, measured using custom software that tracks the sperm.¹⁶ The ratio of VCL after trapping to VCL before trapping is calculated to measure changes in swimming speed as a result of the laser trap. The Student's t-test¹⁷ was used to compare the ratios of sperm held at 15 sec, 10 sec, and 5 sec after data were found to be normally distributed by the Lilliefors test.¹⁷

2.4 Experiment 2—Trapping Power

The goal of this experiment is to determine if the laser trapping power has a significant effect on sperm motility. The laser trap duration is held constant at 5 sec. The effects are tested on sperm from the same pooled dog sperm sample as in the trapping duration experiment. Sperm are trapped at 420 mW, 350 mW, 300 mW, and 250 mW laser power in the specimen plane. The VCL is measured prior to and post trapping as in experiment 1. The Student's t-test¹⁷ is used to compare the ratios of sperm held at 420 mW, 350 mW, 300 mW, and 250 mW after data were found to be normally distributed using the Lilliefors test.¹⁷

2.5 Experiment 3—Correlation of Swimming Force and Swimming Speed

The rotation rate of the motorized mount that holds the polarizer is programmed to produce a linear power decay from maximum (100%) to minimum ($\approx 0\%$) in 10 sec. A maximum trapping power of 366 mW in the specimen plane was used in these experiments. Studies were conducted on sperm samples pooled from four dogs (different than those used in the trap duration and trapping power experiments) as well as on sperm samples from individual dogs that were not pooled.

A sperm of interest is observed for three to six seconds before and after trapping. Once a sperm is trapped, the user initializes the power decay within one second. The moment the sperm escapes the trap, the user halts the power decay, and the escape power is recorded by the computer. Recorded video segments pre- and post-trapping are analyzed by the custom software to calculate the VCL, straight-line velocity (VSL), total distance traveled, and ratio of displacement to total distance traveled. Video of the sperm are independently analyzed by three fertility experts, each blind of the VCL and VSL, who assign each sperm an SOP score (based on the 1–5 scale). Sperm that appear to be swimming in a circular path (those with no forward progression) are given an SOP score of 2 regardless of swimming speed. These sperm were eliminated from the dataset.

The data from the pooled dog sperm sample is organized into a matrix with each row representing a single sperm and each column containing the nonsubjective measurements: (1) VCL pre-trapping; (2) escape laser power (Pesc); (3) swimming displacement pre-trapping; (4) curvilinear distance traveled pre-trapping; and (5) ratio of displacement to total distance traveled. Principal components analysis (PCA) of the data matrix was performed in order to seek hidden relationships between the five measurements.¹⁸ In PCA, the data matrix is linearly mapped into principal component (PC) space after standardizing each column by the estimate of its standard deviation. The data are transformed by

$$\text{new_data}(n,m) = \sum_{i=1}^n \left[\left\{ \text{std_data}(n,i) - \text{mean}(\text{std_data}(:,i)) \right\} * w(i,m) \right],$$

where $w(i,m)$ are the PC weights for a matrix of n rows and m columns and std_data is the standardized dataset. The first two measurements (principal components) of the new dataset contain the majority of the variation in the data (the greatest variance, $\sim 50\text{--}60\%$, by any projection of the dataset lies on the first axis and the second greatest variance, $\sim 20\text{--}30\%$, on the second axis¹⁹). Plotted against one another, they display a more compact distribution of the data as compared to the original measurements.

A supervised classifier using modified SOP scores for training was implemented to determine the most probable hyperplane(s) for separating classes of sperm in PC space. The SOP scores were modified since the number of separable sperm classes was not found to be necessarily equal to the number of SOP classifications, likely due to the subjectivity of the SOP scoring system. Instead, the number of sperm classes was determined by the number of statistically separable

Table 1 Effect of Laser Trap Duration and Laser Power of on Sperm Motility

	Average velocity ratio
5 sec	0.9473, +/-0.18
10 sec	0.9097, +/-0.19
15 sec	0.8602, +/-0.23
420 mW	0.9473, +/-0.18
350 mW	0.9251, +/-0.14
300 mW	0.9475, +/-0.14
250 mW	0.9564, +/-0.13

Note: Average velocity ratios (+/- standard deviation) for various trap durations (constant trapping power, 420 mW) and various trapping powers (constant duration 5 sec). The 15-sec trap duration has the greatest average decrease in VCL post-trapping.

able groups within the SOP scoring system, as assessed by the Wilcoxon paired-sample test.¹⁷ The supervised classifier returns the misclassification error rate, which is the percentage of observations in the dataset that are reclassified with respect to the modified SOP scores. Classified data are remapped into the original data space to understand the physical implications of the determined classes. VCL is plotted against Pesc for each class, and robust linear fitting is applied to seek a trend within each sperm class.

The trained classifier was tested with naïve data consisting of two individual, as opposed to pooled, dog sperm samples (exclusive from the pooled sperm sample set). The classifier first maps the naïve data into PC space using PC weights calculated from the pooled dog. The data are then classified using the pooled data as a training set. Linear regressions of Pesc vs. VCL are fit to each resulting class of sperm. This procedure is also used to test the effects of sample temperature by analyzing individual dog sperm samples at room temperature (approximately 25°C) and at 37°C.

3 Results

3.1 Experiment 1—Trap Duration

A total of 179 dog sperm were trapped and analyzed: $N_{15\text{ sec}}=53$ sperm ($\langle \text{VCL pre-trap} \rangle = 102 \mu\text{m/sec}$, range $[60.7\text{--}151] \mu\text{m/sec}$), $N_{10\text{ sec}}=77$ ($\langle \text{VCL pre-trap} \rangle = 110 \mu\text{m/sec}$, range $[37.4\text{--}200] \mu\text{m/sec}$), and $N_{5\text{ sec}}=49$ ($\langle \text{VCL pre-trap} \rangle = 104 \mu\text{m/sec}$, range $[39.8\text{--}174] \mu\text{m/sec}$). Average velocity ratios of VCL post-trapping to VCL pre-trapping for various trap durations were determined (Table 1). Figure 2 plots sperm velocity ratios for the three trapping durations. The ratio of “1” is emphasized in order to distinguish the sperm that swam out of the trap at a higher velocity than before trapping from those that swam out of the trap at a lower velocity. The results of the Student’s t-test that compare the means of the sperm velocity ratios are also listed in Fig. 2. A trap duration of 15 sec resulted in a greater decrease in VCL than 10-sec or 5-sec durations (at 420 mW laser

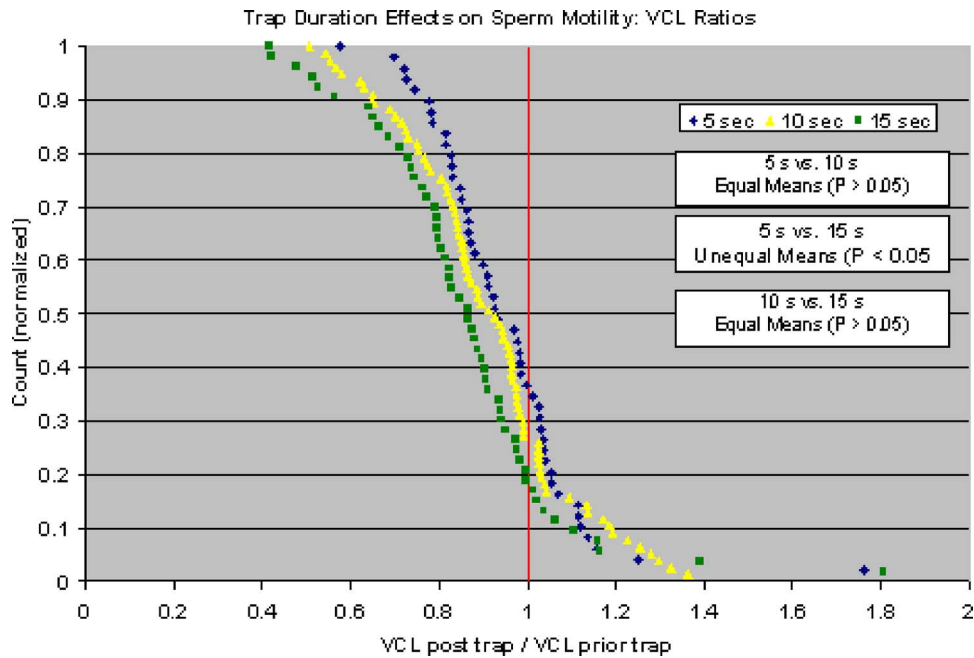


Fig. 2 Experiment 1—Effects of Trap Duration: VCL ratios for the three trap durations tested (15 sec, 10 sec, 5 sec) at constant power (420 mW). The less time sperm are exposed to the trap, the closer the VCL ratio is to unity (less effect on sperm motility). The results of the Student’s t-test (*P*-values) are listed.

power). Average VCL decrease for trap durations of 10 sec and 5 sec were negligible and statistically equivalent.

3.2 Experiment 2—Trapping Power

A total of 195 dog sperm were trapped and analyzed: $N_{420\text{ mW}}=49$ ($\langle\text{VCL pre-trap}\rangle=104\ \mu\text{m/sec}$, range [39.8–174] $\mu\text{m/sec}$), $N_{350\text{ mW}}=49$ ($\langle\text{VCL pre-trap}\rangle$

$=108\ \mu\text{m/sec}$, range [41.7–185] $\mu\text{m/sec}$), $N_{300\text{ mW}}=51$ ($\langle\text{VCL pre-trap}\rangle=78.2\ \mu\text{m/sec}$, range [85.5–140] $\mu\text{m/sec}$), and $N_{250\text{ mW}}=46$ ($\langle\text{VCL pre-trap}\rangle=91.6\ \mu\text{m/sec}$, range [42.0–165] $\mu\text{m/sec}$). Average velocity ratios for various trapping powers are listed in Table 1. Figure 3 plots sperm velocity ratios for all four trapping powers. The data at all four trapping powers were found to be

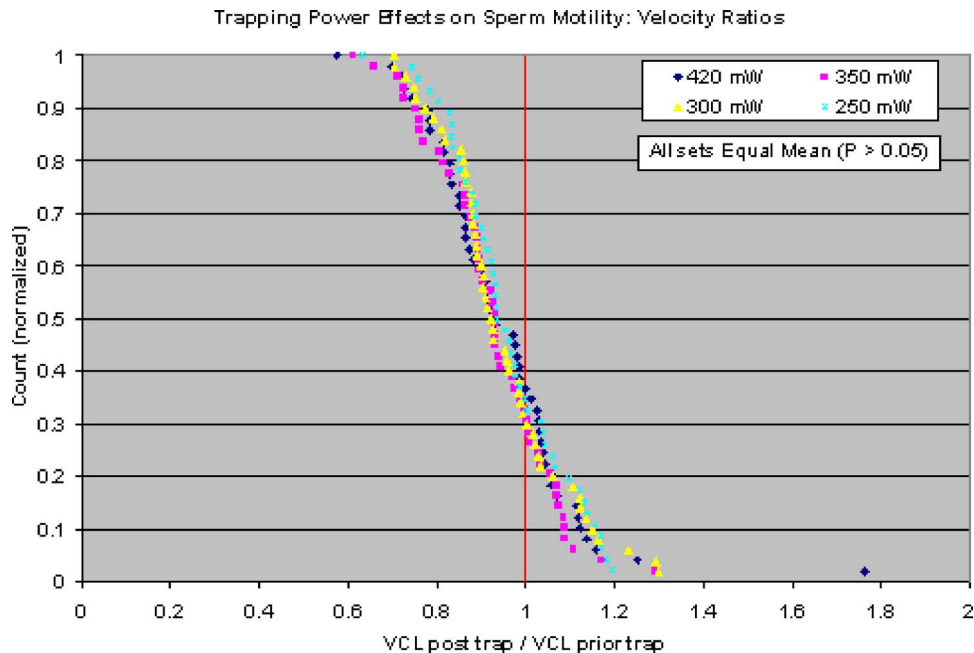


Fig. 3 Experiment 2—Effects of Trapping Power: VCL ratios for the four trapping powers tested (420 mW, 350 mW, 300 mW, 250 mW). All VCL ratios are close to unity.

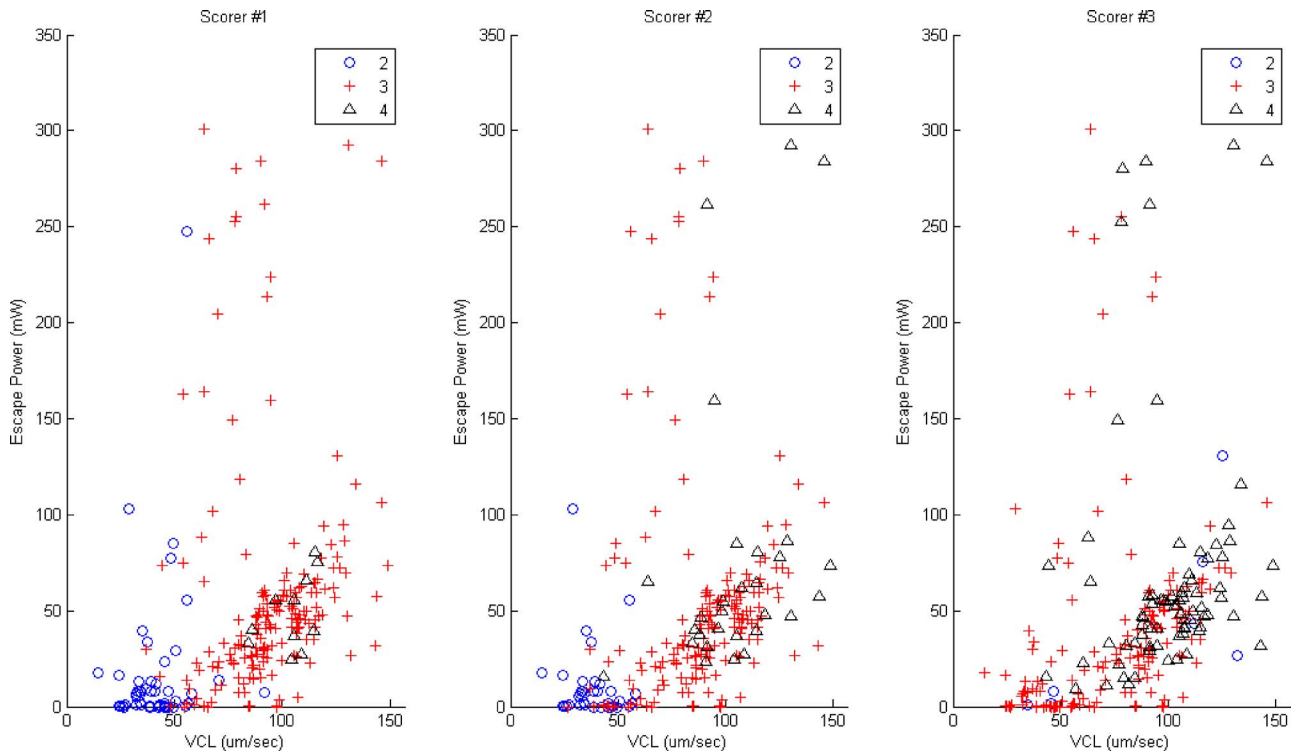


Fig. 4 Experiment 3—Discrepancy between SOP score assignment between individuals: Pesc vs. VCL for the pooled dog sperm sample with scores assigned by scorers #1, #2, and #3 (left to right).

statistically equivalent ($P > 0.05$), and the average decrease in VCL post-trapping is considered negligible. Thus, a 10-sec power decay beginning at 420 mW or less should not significantly affect sperm motility.

3.3 Experiment 3—Correlation of Swimming Force to Swimming Speed

Figure 4 demonstrates the subjective nature of SOP scores. Pesc (mW) is plotted against VCL ($\mu\text{m}/\text{sec}$) for the pooled dog sperm dataset (excluding circle swimmers). The data are labeled according to the SOP scores assigned by each of the

three qualified sperm scoring experts. Variability in assigned SOP score is evident. As expected, a linear relationship between swimming force and Pesc was found with $R^2 \approx 0.9$ using a robust linear regression. However, doing so is contrary to a qualitative analysis of the data that suggests there may be intrinsic groupings, each with their own Pesc to VCL relationship. For example, potential groupings could be a high escape power group and a low escape power group or a slow group and a fast group with high Pesc outliers.

PC analysis consolidated 73% of the variation in the data to within the first two principal components. Table 2 lists the

Table 2 Principal Component Analysis

	PC 1	PC 2	PC 3	PC 4	PC 5
% Variability explained	52.0663%	21.1449%	15.9962%	10.2293%	0.5633%
VCL	-0.48	0.11	-0.04	0.87	0.04
Pesc	-0.26	0.59	0.74	-0.18	-0.001
Disp	-0.58	-0.23	-0.10	-0.33	0.71
Dist Pt2Pt	-0.49	-0.55	0.23	-0.16	-0.62
Disp/Dist Pt2Pt	-0.36	0.54	-0.62	-0.28	-0.34

Note: Percentage of variability each PC is responsible for and the PC weights for each measurement that transform the original data of pooled dog sperm samples into new data (PC space).

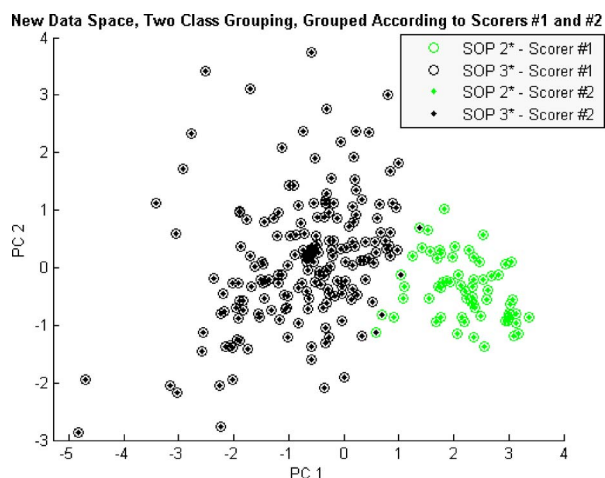


Fig. 5 Experiment 3—Data Variation Maximization: Pooled dog sperm data set in PC space—optimum group division shown for two classes. Scorer #1 plotted as circles, scorer #2 plotted as dots, both showing SOP 2* group in green and SOP 3* group in black.

percent variability accounted for by each PC as well as the PC weights. Figure 5 plots PC1 vs. PC2 for the pooled sperm data.

To test uniqueness of the SOP scores, Wilcoxon paired-sample tests¹⁷ for equal median (5% significance level) were performed on Pesc distributions of sperm grouped by SOP score, for each scorer. Table 3 summarizes the number of significantly separable groups each scorer distinguished. Two of the three scorers uniquely distinguished at most two groups of sperm, consisting of “slower” and “faster” swimmers. For the purpose of classification in PC space, modified SOP scores were constructed based on these findings: “slower” = SOP 2* and “faster” = SOP 3* (Table 3 maps SOP scores to modified SOP scores for each scorer).

Figure 5 plots the pooled dog sperm data in PC space labeled by their modified SOP scores for scorers #1 and #2. As can be seen, the region of support lies nearly along a straight line. A supervised classifier with a linear hyperplane was found to yield the lowest misclassification error rate (7.44%). A classifier based on scorer #3 had a misclassification error rate of 28.05% (not shown). Subsequent data analysis is based only on classifiers trained from scorers #1 and #2.

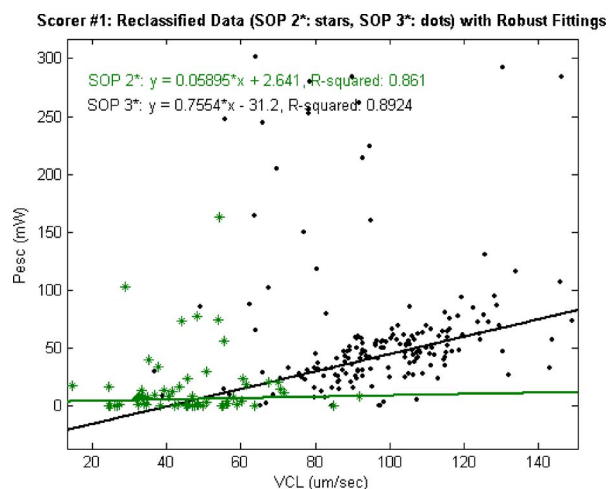


Fig. 6 Experiment 3—Classified data with regressions: Pesc vs. VCL for pooled dog sperm sample is plotted according to the reclassification for scorer #1 with robust linear regressions applied to each sperm class.

Figure 6 plots the data of Fig. 4 labeled by classifier output. Linear regressions with robust fitting for each group (SOP 2*, SOP 3*) are shown for scorer #1. Table 4 lists the slopes (m), y -intercepts, and R^2 -values for linear regressions applied to the original dataset and applied to the classified groups, by scorer. Regressions were compared using the Student’s t -test.¹⁷ Regression parameters of the original data were statistically different from the SOP 2* and SOP 3* groups, casting doubt on the validity of regressing the entire dataset. Interestingly, SOP 2* slopes showed little to no relationship between Pesc and VCL (mean value was 0 or 0.02 for scorers #1 and #2, respectively). Both scorers revealed an SOP 3* group with equivalent nonzero slopes. This suggests the SOP 3* group has a linear Pesc to VCL dependence.

Of interest are the outlying sperm that escape the trap at higher powers than the majority of the sperm analyzed. They were neither identifiable by their SOP score (see Fig. 4), nor as an independent group using PCA and supervised classification. One possibility is that these outlier sperm are all from one specific dog within the pooled sample which has stronger-swimming sperm. This theory is tested by repeating the experiment using the single-dog sperm samples. Figure 7 plots

Table 3 Defining Sperm Classes

	SOP 2 vs. SOP 3	SOP 3 vs. SOP 4	# Distinct Classes	SOP 2*	SOP 3*
Scorer #1	Unequal medians ($P < 0.05$)	Equal medians ($P > 0.05$)	2	SOP 2	SOP 3 and 4
Scorer #2	Unequal medians ($P < 0.05$)	Unequal medians ($P < 0.05$)	3	SOP 2	SOP 3 and 4
Scorer #3	Equal medians ($P > 0.05$)	Unequal medians ($P < 0.05$)	2	SOP 2 and 3	SOP 4

Note: The Wilcoxon paired-sample test applied to pooled dog sperm sample shows which scorers distinguish a separation between sperm of different SOP scores and the overall number of distinct classes each scorer can differentiate. The last two columns show how each sperm’s original SOP score is reassigned to fit the new SOP* classes.

Table 4 Unclassified vs. Classified Data

	Original data	Scorer #1: SOP 3*	Scorer #1: SOP 2*	Scorer #2: SOP 3*	Scorer #2: SOP 2*	Individual dog sperm (SOP 2*)	Individual dog sperm (SOP 3*)
Slope (m)	0.6430	0.7554	0.0586	0.7526	-0.0244	0.0496	0.4215
v-int.	-20.65	-31.20	2.64	-30.93	4.79	-0.0075	-11.48
R ² value	0.8977	0.8924	0.8610	0.8952	0.893	0.8307	0.8567

Note: Comparison of regression values (slope, y-intercept, and R^2) for the four sets of regressions applied to the pooled dog sperm data (original, reclassified for scorer #1, reclassified for scorer #2) and the individual dog sperm data.

Pesc vs. VCL for the individual dog sperm samples (dog 1 and dog 2 tested on separate days) superimposed with the pooled dog sample data. Both individual dog sperm samples exhibit two groups of sperm, slower and faster swimming, as well as outlying sperm that escape the trap at higher trapping powers. Using the Wilcoxon rank sum test, VCL and Pesc of dog 2 were found to have equal medians ($P > 0.05$) between days one and two.

The individual dog sperm data were transformed into PC space by the PC weights calculated with the pooled dog sperm data. Subsequent classification (trained on the pooled dog data) had a misclassification error rate of 1.36%. Linear regression of the SOP 2* group shows a near-zero slope while regression of the SOP 3* group finds a linear increase in swimming force with swimming speed. Table 4 summarized the regression results.

Sperm velocity distributions were found to be of equal median ($P > 0.05$) for a sample analyzed at room temperature and at 37°C. Regressions fit to escape power vs. VCL are found to be statistically equivalent ($P > 0.05$).

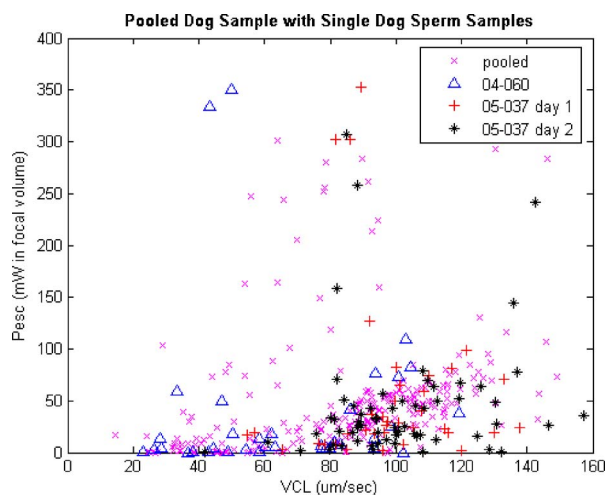


Fig. 7 Experiment 3—Comparison of individual samples with pooled samples: Pesc vs. VCL for individual dog sperm samples referenced with pooled dog sample. Dog 1 in triangles, dog 2 (day 1) in pluses, dog 2 (day 2) in asterisks, and pooled dog in x's.

4 Discussion and Conclusions

A negligible decrease in swimming speed post-trapping was observed at all tested trapping powers (420 mW, 350 mW, 300 mW, and 250 mW) for a 5-sec trap duration. However, a 15-sec trap duration at 420 mW was found to statistically impede motility. Not surprisingly, there was a nontrivial relationship between laser power and trap duration with respect to motility. However, we found that at 420 mW laser power, 10-sec and 5-sec trap durations were equivalent and negligible with respect to sperm motility. Accordingly, we chose 10-sec duration for subsequent experiments in order to soften the pace of laser decay to better detect the precise moment of escape. Similar studies on human sperm found that slower-swimming sperm (1 to 60 $\mu\text{m}/\text{sec}$) were unaffected by the laser trap for durations up to 30 sec at a power of 1 W in the focal volume using a 1064-nm Nd:YAG laser,⁸ while in this study comparatively faster swimming dog sperm ($\sim 105 \mu\text{m}/\text{sec}$ average, with a maximum swimming speed of 200 $\mu\text{m}/\text{sec}$) were affected in as little as 15-sec trap duration using less laser power (maximum of 420 mW). This suggests either species-specific laser sensitivity, or faster-swimming sperm are more sensitive to the laser trap. Optical trap exposure and power tolerances could be a function of species phenotype, specifically sperm head geometry and biophotonic properties. Future studies will investigate species dependence in the response to laser trapping.

Escape power plotted against VCL suggests a complicated relationship. The inclusion of standard SOP scores neither assisted in categorizing the data into nonoverlapping domains, nor provided a deeper understanding of the data. The SOP scores were found to be neither independent nor mutually exclusive, requiring a modification of the SOP scores into two new SOP scores (SOP 2* and SOP 3*). These modified SOP scores were used to train a classifier operating on the first 2 PCs of motility measurements. This analysis revealed at least two distinct classes of sperm separated along the VCL axis of a Pesc vs. VCL plot. Linear regressions applied separately to the two sperm classes were found to be significantly different than the regression of the entire dataset.

Two conclusions can be drawn from these results. The first is that the SOP 2* and SOP 3* sperm have significantly different dependencies of Pesc on VCL. Pesc for the SOP 2* sperm was not found to increase with swimming speed. This

could be either a biological phenomenon or an experimental bias since it is difficult to pinpoint the precise moment that a slow-swimming sperm escapes from the trap. Sperm in this category require further investigation with an incrementally decreasing step function in laser power, rather than a continuous linear downward ramp. In the SOP 3* group, Pesc increased linearly with swimming speed. This is consistent with the relationship between swimming force and viscous resistance for sperm swimming at constant thrust. Experiments are currently being conducted on other species to determine if parameters of classification and linear regression correlate to species type.

The second conclusion is that Pesc is necessary but not sufficient to distinguish SOP 2* from SOP 3* sperm. Figure 5 shows that variation in the data is primarily along the PC 1 axis, where the Pesc weight is minimal. However, the hyperplane dividing the two groups is not parallel to the PC 2 axis. Therefore, the PC 2 dimension nontrivially determines the sperm class. In this system, escape power is a critical parameter in sperm classification since it has the largest weight in PC 2, which represents 21.14% of the variability in the data. Therefore, it is concluded that the effective use of escape power as a variable to assess sperm motility requires that it be used in conjunction with other established parameters (velocity, displacement, etc.) to characterize the sperm. This combination results in a more comprehensive evaluation of overall sperm quality.

Results from individual dog sperm samples negated the notion that outlier sperm were artifacts of experimental technique. Individual dog data also validated the classifier, which was trained on the pooled dog sperm sample. Classifier validation showed low error rates and an equivalent separation of sperm classes between the training (pooled) and testing (individual) data suggesting we have observed a general phenomenon, and not just one confined to the pooled dog sperm.

The individual dog sperm analysis also confirmed the presence of "outlier" sperm and negated the notion that outlier sperm in the pooled dog sperm data represented the sperm of one single dog. The existence of outliers may point to a fundamental physiological distribution in sperm phenotype. It is possible that outlier sperm are in a state of increased physiological energetics (such as at a higher rate of ATP production and/or consumption) and may respond to physical/chemical barriers in a distinctly different manner than the rest of the sample. Fluorescent probes capable of measuring energetics and metabolism should "shed light" on the outlier sperm. If there is a subgroup of sperm within a semen sample that exhibits higher motility characteristics, their separation and study could be of considerable value in both basic and applied infertility research. In conclusion, in this paper we have described the use of a laser optical trap to measure the swimming force(s) of a large number of individual sperm in a semen sample, thus expanding the quantitative parameters available for assessment of sperm motility and semen quality.

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