Supporting Information for

Rheotaxis Quality Index: A New Parameter That Reveals Male Mammalian in vivo Fertility And Low Sperm DNA Fragmentation

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1- Flow rate injection using hydrostatic pressure

We used particle image velocimetry (PIV) analysis of the egg-yolk particles in the channel with 20 frames per second to show that as the height of the injection reservoir increases, mean velocity in the channels increases linearly.

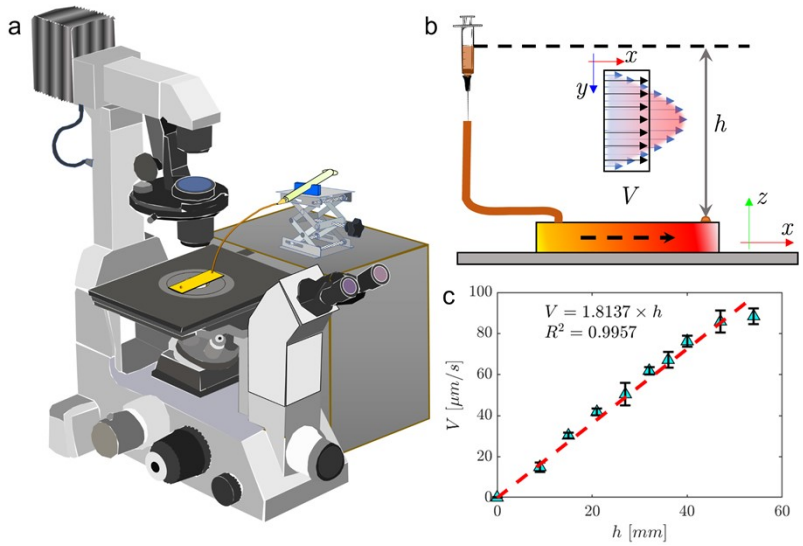

Figure S1. (a) The experimental setup. (b) The mean velocity in the cross section (V) of the channel and the corresponding height (h) for injection that flow rate. (c) There is a linear relationship between V and h in the biologically relevant velocities.

2- Measurement of flow rate in the channel using PIV at the probe span

We have calculated the flow rate based on the PIV measurement and comparison between the finite element method (FEM) simulations. The profile of the velocity at the span of the probes was used as the region of interest for PIV analysis. Figure S2a-c shows the result of velocity magnitude on the line of X = 650 µm for three inlet height. The profile is depicted for 5-minutes experiments every 60 seconds. The mean velocity profile was then used for calculation of the flow rate.

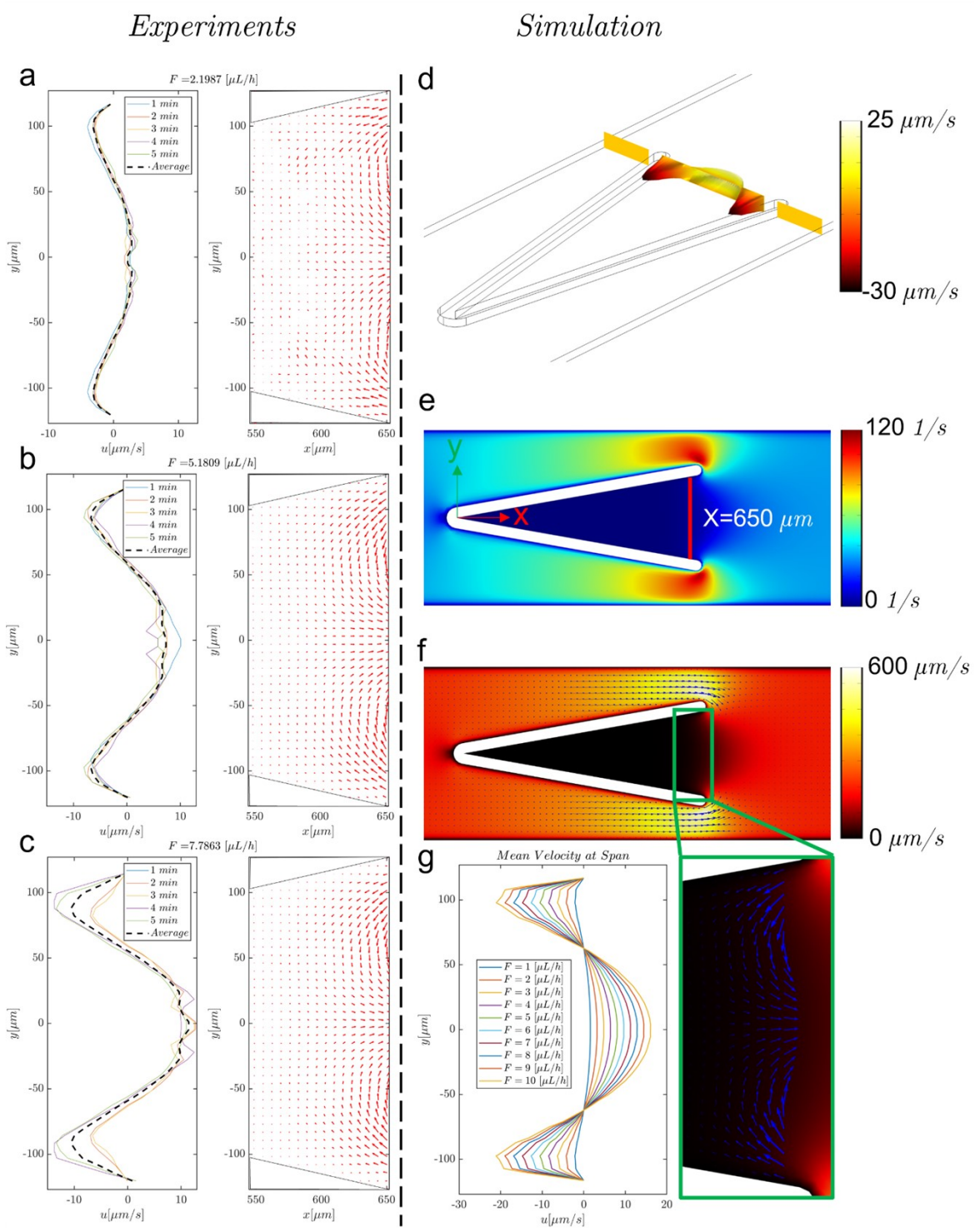

Figure S2. (a-c) experimental measurements of velocity vectors and x-component of velocity on the line of x=650 μm, related to PIV of the egg-yolk particles at the span of the probe 20°. (d-g) Simulation results of FEM analysis for velocity profile at the span of the probe for the flow rate of F = 7 μL h^{-1}

Since 50 frames were captured for evaluation of the signal, 50 images were fed into PIV lab code and a rectangular area of width 100 µm and height 300 µm was chosen with the right side coinciding with X = 650 µm (corner of the probe was chosen as origin in Figure S2e). Using the difference of consecutive images, PIVlab resulted in 49 flow fields which were averaged to calculate the experimental velocity field at the span of the probe.

For evaluation of flow rate in the channel within the same video captured for the signal, we used the fact that velocity profile in Newtonian fluids is linearly affected by the flow rate. We measured the mean velocity profile at the probe's span (on the line of X = 650 µm as depicted in Figure S2e) and then compared the experimental velocity profile to the theoretical velocity profile extracted from the FEM simulations (Figure S2g). The comparison was performed using the least squares algorithm. Taking u_t(F,y) as the theoretical velocity profile and u(y) as the experimental velocity profile, the following expression was calculated for g(F) from F = 1 to 10 µL h^{-1} and linear interpolation was used to find F for which g(F) = 0.

$$g(F) = \Sigma_y (u_t(F,y) - u(y))^2$$

Where Σ_y means sum over y.

For the flow rates of 1-5 µL h^{-1} the flow rate does not change significantly over the experiment time but for higher flow rates such as 7.8 µL h^{-1} shown in the Figure S2, the variations increase.

3- Rheotaxis zone and mean rheotaxis velocity

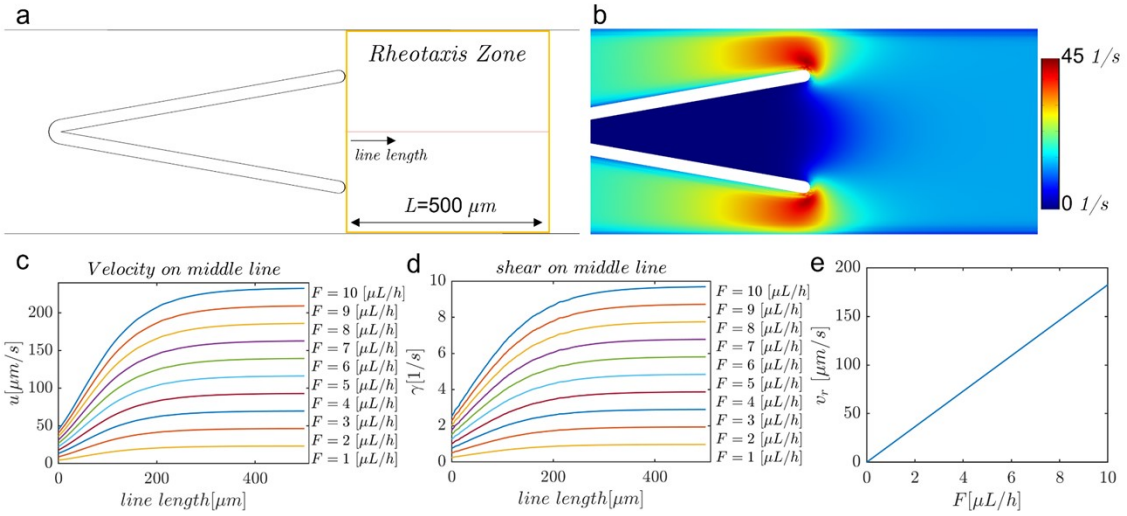

Figure S3. Rheotaxis zone and mean rheotaxis velocity. (a) Rheotaxis zone is a cuboid at the end of the probe with the length of L = 500 μm. (b) simulated shear rate contour on a plane 9 μm from the top surface for the flow rate of 5 μL h^{-1}. (c) velocity on the red middle line depicted in (a) which is 9 μm from the top surface. (d) shear rate on the middle line. (e) mean rheotaxis velocity (v_r) with respect to the flow rate.

4- Signal-number curve resolution analysis

In this section, a relatively simple statistical analysis is used based on the t-test to define at which point the signal-number curve of the 20° probe becomes saturated statistically.

We chose to treat each replication individually and refer to the 49 signal instances for each average signal. Further in SI Section 7 we show the evidence that those 49 signal instances are scattered normally around the mean, therefore for the signal-number to be saturated means that we should find a number N above which the average signal values are no longer statistically significantly different.

$$T = \frac{S_{N+\Delta N} - S_N}{\sqrt{\frac{2\sigma^2}{n}}}$$

For this equation, n = 49 and σ is ~0.3 (associated with inherent noise of the system in our experiments) and $S_{N+\Delta N} - S_N = \Delta S \sim dS = A.b.N^{b-1}\Delta N$ for which ΔN is the number of sperm that should be increased to get to the ΔS. For the fitted parameters,

$$T = 3.192N^{-0.38}\Delta N$$

For the significance level of α = 0.05, T must be more than 1.9673, so:

$$N^{0.38} < 1.54\Delta N$$

If we define $1/\Delta N$ as the resolution of the method then the number above which saturation occurs, can be calculated based on this resolution. Figure S4 shows the dependency of the resolution on the number of sperm in the probe. The resolution decreases as the number of sperm in the probe increases. The first four sperm will be discernable one-by-one as $\Delta N = 1$. From N = 4 to 20, resolution is 2 sperm; meaning that the sperm will only be discernable two-by-two. Resolution further decreases to 3 sperm within the range of N = 21 to 57 and from 57 to 120 the resolution would be 4 sperm at a time at which point the maximum signal is S = 6. This signal is the maximum signal reached in the experiments regarding fertility (SI Section 10). Increasing the number from

121 to 216 will reduce the resolution to 5. The maximum S in this paper is S = 15.6 (Movie S2) which happens at a resolution of 7 sperm at a time and at N ~520 sperm (Figure 5c).

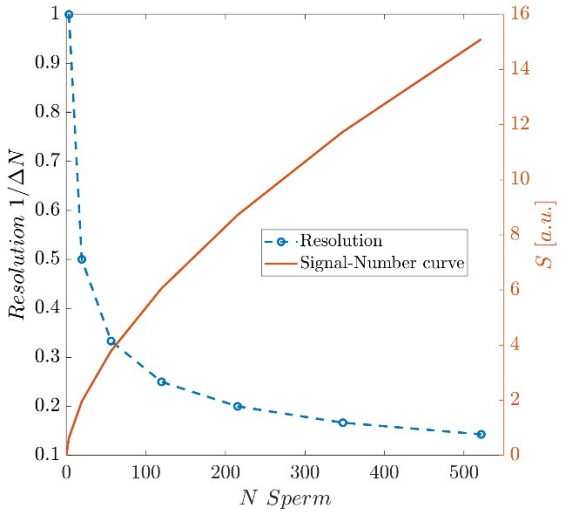

Figure S4. Resolution of the sperm detection in the probe 20° for various sperm numbers along with the fitted signal-number curve. Resolution decreases to 7 sperm up to N = 520.

5- Signal over time for the wide corner angles of θ = 80° and 130°

The same flow rate of F = 3.95 μL h^{-1} used in this design to be comparable to the Figure 3 results.

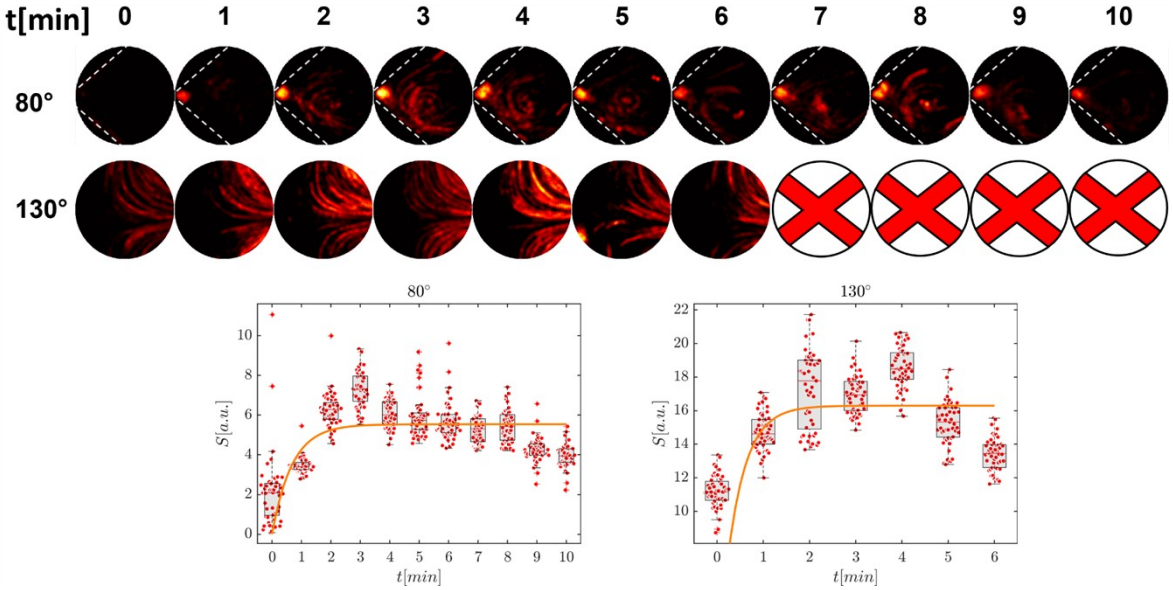

Figure S5. Signal over time for other probe angles. As the corner angle increases the debris and egg-yolk particles also enter the frame of sperm and interfere with the signal. The streamlines of

the particles are visible from the contours of both θ = 80° and 130°. Because of the wide angle (θ > 50°) in these designs the sperm that undergo rheotaxis and swim upstream could not be collected in the corner and readily swim back to the main stream. Whereas in acute angles sperm will be trapped in the probe for the duration of the tests (5 minutes). For θ = 130° experiment was stopped at t = 6 minutes since there was no meaningful representation of the signal.

6- Head Rolling Frequency (HRF)

For the HRF the intensity of the head of the sperm was monitored. If the normalized intensity of the head jumps over 1.0 and reduces, the head has rolled once. For example, for the transition from t = 1.08 s to 1.13 s the sperm head intensity changes from 0.53 to 1.33 and reduces back to 0.56 at 1.28 s. This is marked with a star in the Figure S6a. The same pattern occurs t = 2.31 s. The duration between the consecutive occurrence of rolling is the period of rolling which we reciprocated the mean value of periods to calculate HRF. This algorithm is the same as others used in this field.[1]

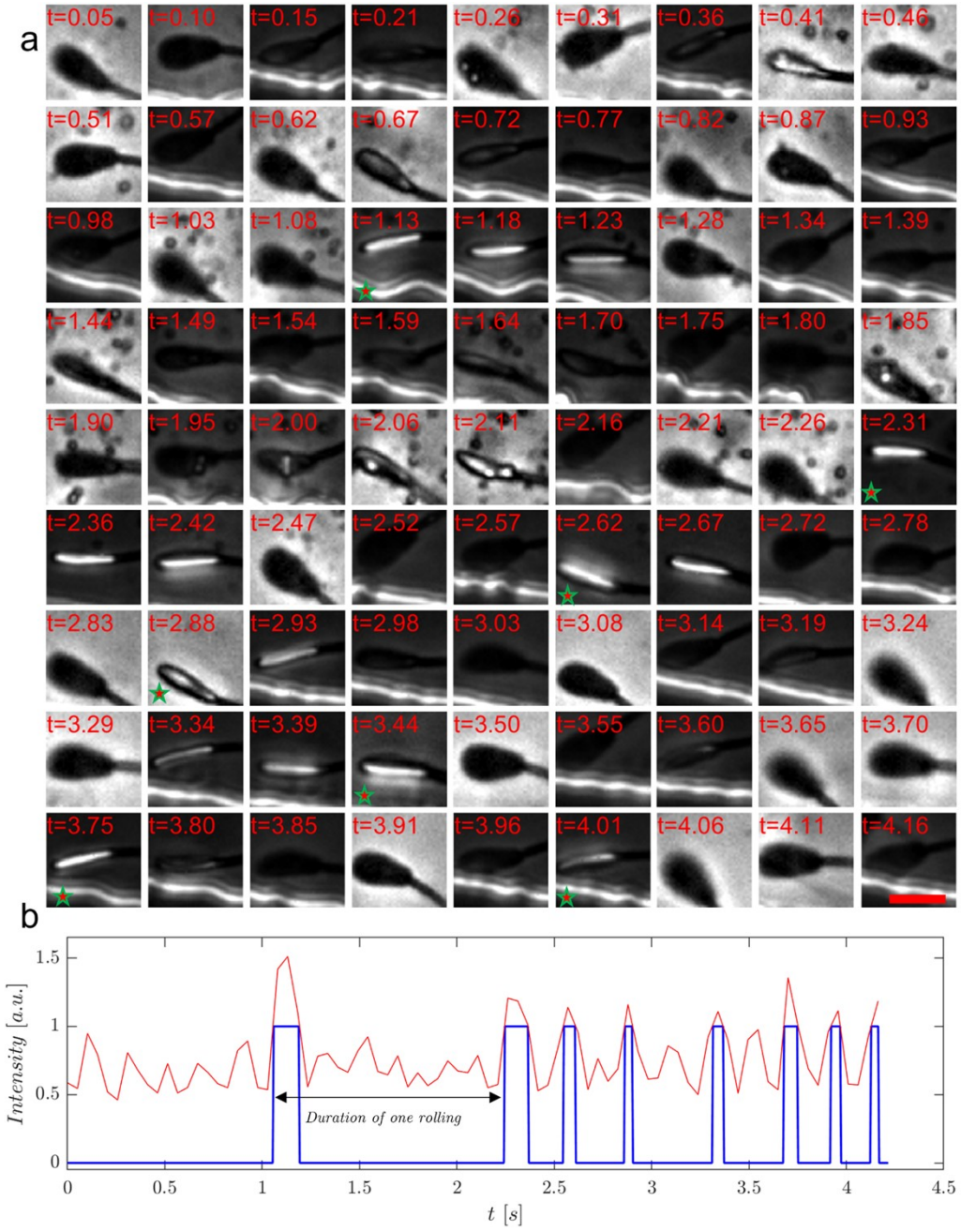

Figure S6. (a) HRF measurement based on the intensity of the head while sperm tracking (a) head images over time in seconds. The stars on some images denote the incidence of the rolling in the sperm head. The scale bar is 10 µm. (b) Normalized head intensity over time. Rolling occurs if normalized head intensity increases over 1 and reduces back.

7- Fast Fourier Transform (FFT) of the Signal

Signal over time for 1 to 4 sperm in the probe. FFT of the signal specifies the head rolling frequency of the sperm in the probe when there is only one sperm. As the number increases to 2, the FFT no longer bears any meaningful peak. For example, in this case the Figure S7a is related to the first sperm and shows the rolling frequency as 9.2 Hz. Although at Figure S7b another sperm was trapped in the same probe along with the first sperm the signal does not show any peaks at the 9.2 Hz. Another point is that Figure S7a shows a bimodal distribution at S = 0 and 0.48 which is due to the rolling effect on the signal (resembling an on-off signal with the aforementioned frequency). But as the number increases the signal distribution becomes more like a normal distribution and standard deviation becomes more like white noise. Since the head rolling frequency of the sperm in the trap are not equal or in synchronization, the signal over time could not distinguish between multiple sperm. Needless to say, the mean signal follows the signal-number power law despite that signal here is taken over a 15-seconds period.

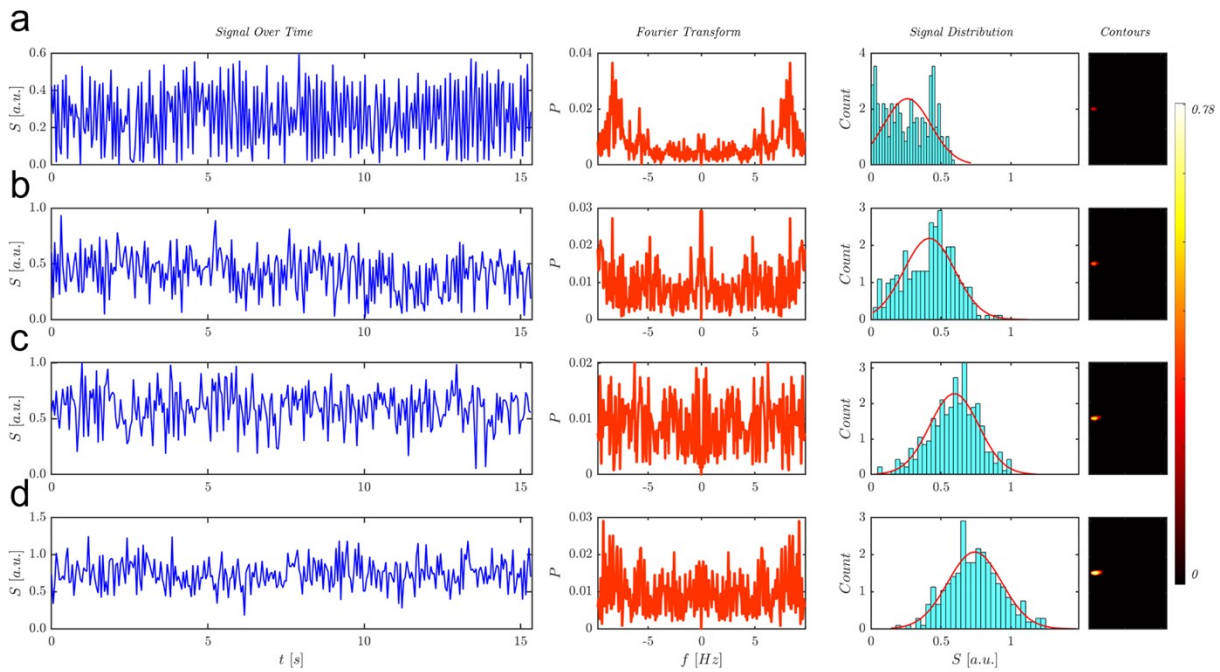

Figure S7. Signal and frequency analysis. (a-d) Signal of first to forth sperm trapped in the 20° degree probe. The figure contains signal over time, FFT results of the same signal, signal distribution along with fitted normal distribution and the contours of the same signal in the 15-seconds period.

8- Characteristic Signal-Flow rate-Concentration surface

To give a more general characteristic description of the outcome of the method, we can combine variation of the signal with respect to concentration and flow rate assuming the signal as a complete differential.

$$dS = \frac{\partial S}{\partial C} \times dC + \frac{\partial S}{\partial F} \times dF$$

We know that $\partial S/\partial C = \alpha_C$ and $\partial S/\partial F = -2\gamma^2 F \alpha_F e^{-(\gamma F)^2}$. By substituting these in the equation above, we can solve for S as a function of C and F as in the equation below.

$$S = \alpha(C - C_{Cr})\, e^{-(\gamma F)^2}$$

By fitting this surface to 20 data points (that include the data in Figure 5a, b and 9 other experiments) we get $\alpha = 4.26\ mL\ M^{-1}$, $C_{Cr} = 0.676\ M\ mL^{-1}$, and $\gamma = 0.2853\ h\ \mu L^{-1}$ with R^2 = 0.925. We can substitute S from the power law in the above equation.

$$AN^b = \alpha(C_{min} - C_{Cr})\, e^{-(\gamma F)^2}$$

For trapping at least one sperm in the corner we must set $N = 1$ which results in the cyan curve in Figure 5c or from equation above

$$C_{min} = A/\alpha\ (\ e^{(\gamma F)^2} + C_{Cr})$$

As F increases, C_{min} that is needed for trapping at least one sperm, increases exponentially so that at F = 7.5 μL h^{-1} we need a sample of C_{min} = 7.18 M mL^{-1} for trapping only one sperm, yet at F = 8 μL h^{-1} (6.6% increment from 7.5) C_{min} increases to 13.5 M mL^{-1} (88.0% increase).

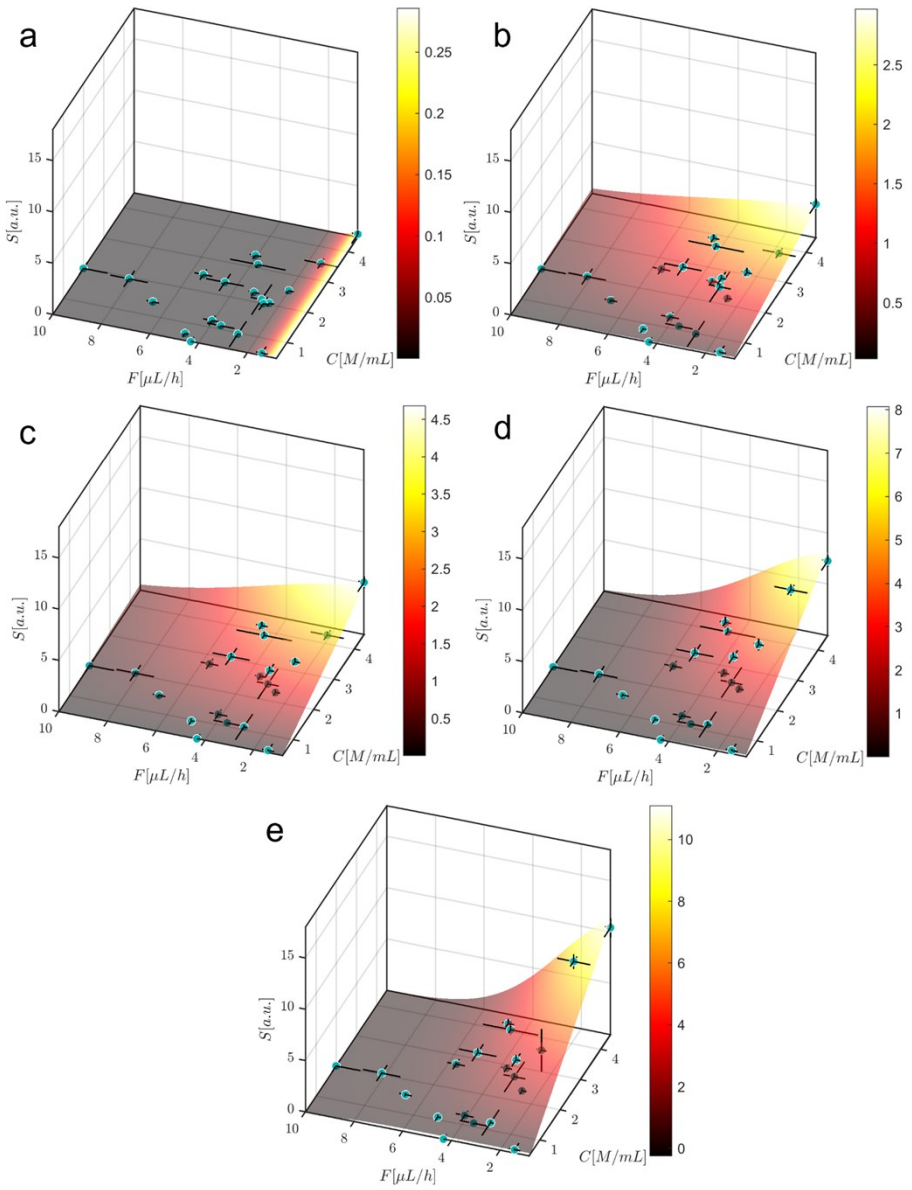

Figure S8. evolution of characteristic curve through time at 1-minute intervals shown in (a-e) chronologically.

We can predict the signal using VAP distribution. Figure 5e shows the VAP distribution of the bull sample used for the characterization of the SFC surface. The probability of sperm being trapped in the probe is the multiplication of the probability of sperm with VAP > v_r by the efficiency of trapping (η). The total number of trapped sperm in 5 minutes (t) is equal to this probability multiplied by the total number of motile sperm (N_M) which is the volume of sample at the span of the probe plus the volume that is brought in by the injection of sample multiplied to the motile sperm concentration.

$$N_M = \eta p\{v > v_r\} \times (V_0 + Ft)C$$

Using the signal-number power law we have,

$$S = A(\eta pC(V_0 + Ft))^b$$

Because many of the sperm do not enter the rheotaxis zone, a small number of motile sperm become trapped in the probe (less than 1 %) compared to the total motile sperm. For η = 0.8 %, C= 2.45 M mL^{-1}, V_0 = 0.0075 μL (corresponding to 500 μm from the span) and VAP from CASA, the black curve in Figure 5f can be calculated.

9- DFI of the 16 bulls with their SCR

Figure S9 illustrates the red and green Signal distributions of sperm heads in the AO test.

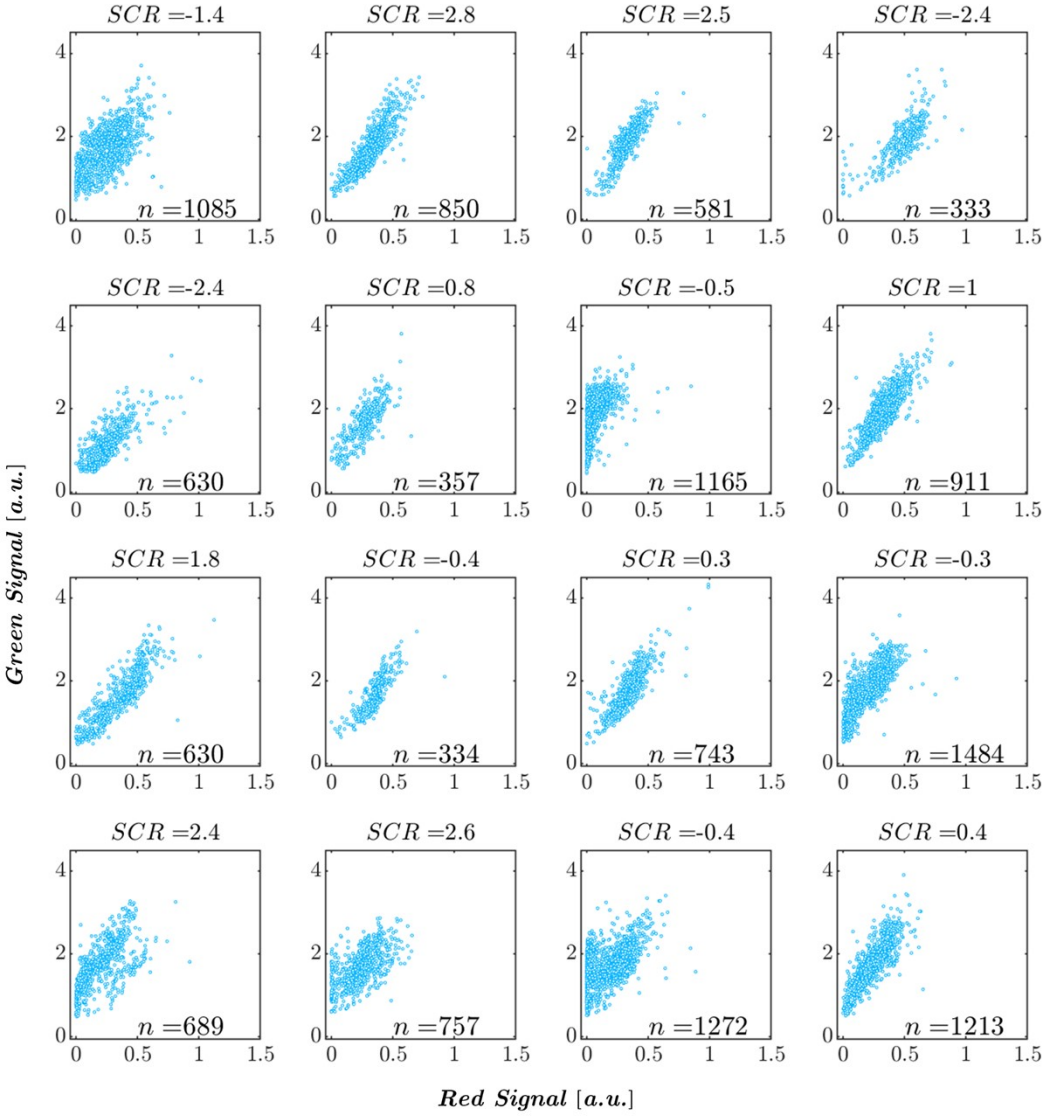

Figure S9. Normalized red and green signals from the AO test. A minimum of 333 sperm were counted for DFI depending on the concentration of semen. If the concentration of the sample was high, more sperm were analyzed.

10- RHEOLEX of the 16 bulls for various flow rates

Figure S10 shows the value of RHEOLEX from the aforementioned 16 bulls. Since the viscosity of semen from males differs from each other, the same inlet reservoir height does not result in the same flow rate for all. But for the purpose of comparison, we should have the RHEOLEX for each of the bulls at the same flow rates. We proved that signal has an exponential decay relationship with the square of the flow rate. Therefore, $S = C_1 e^{-C_2 F^2}$ and thus $ln\,(S) = k - C_2 F^2$. This means that the logarithm of S and F squared has a linear correlation. Blue lines in the Figure S10 are calculated based on this linear relationship. This way, we are able to compare RHEOLEX from the same flow rates.

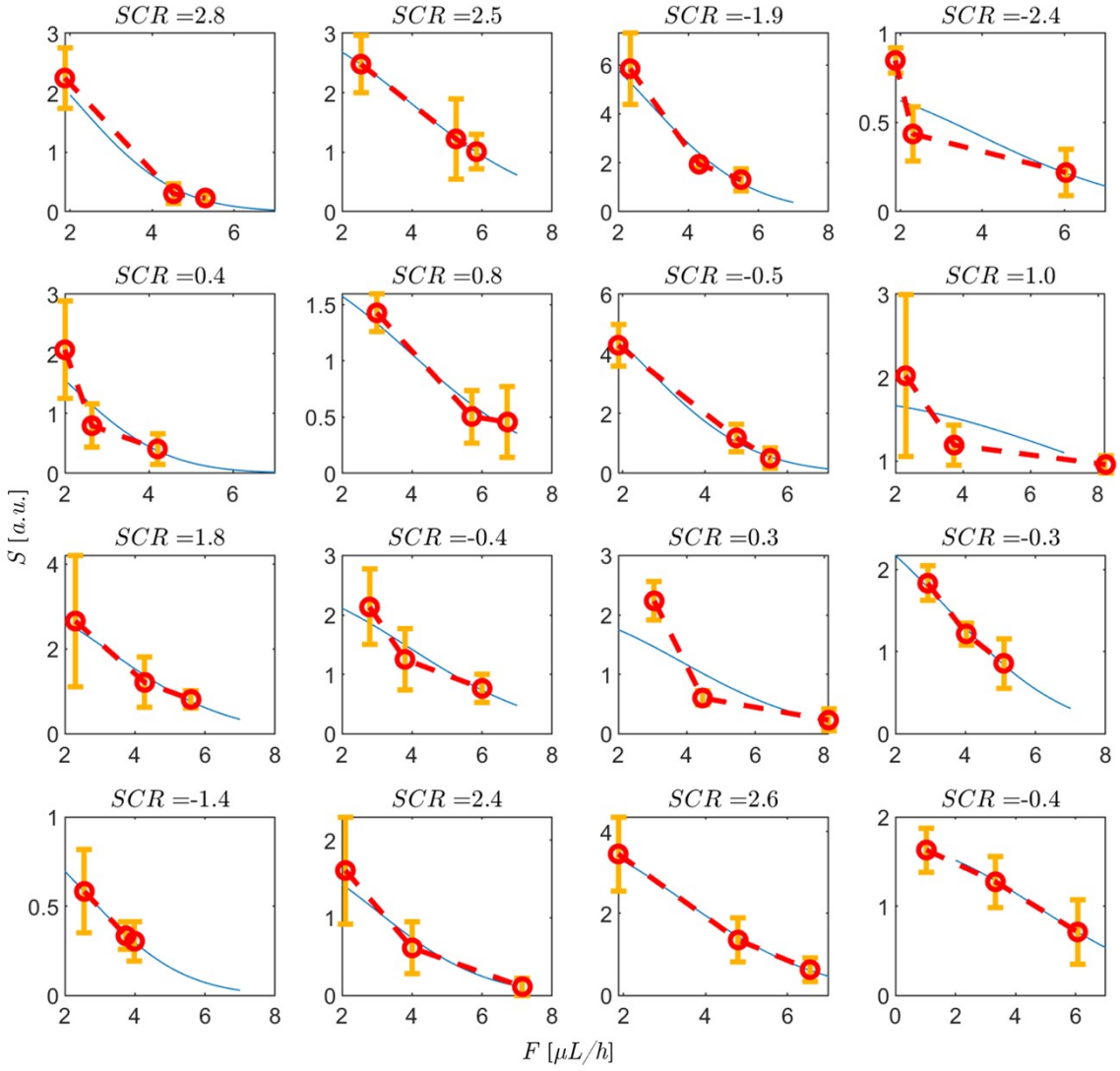

Figure S10. RHEOLEX with respect to flow rate for various bulls. The SCR values are on the top of each figure. Blue lines represent fitted curves based on exponential decay. Red lines represent experimental data of measuring RHEOLEX.

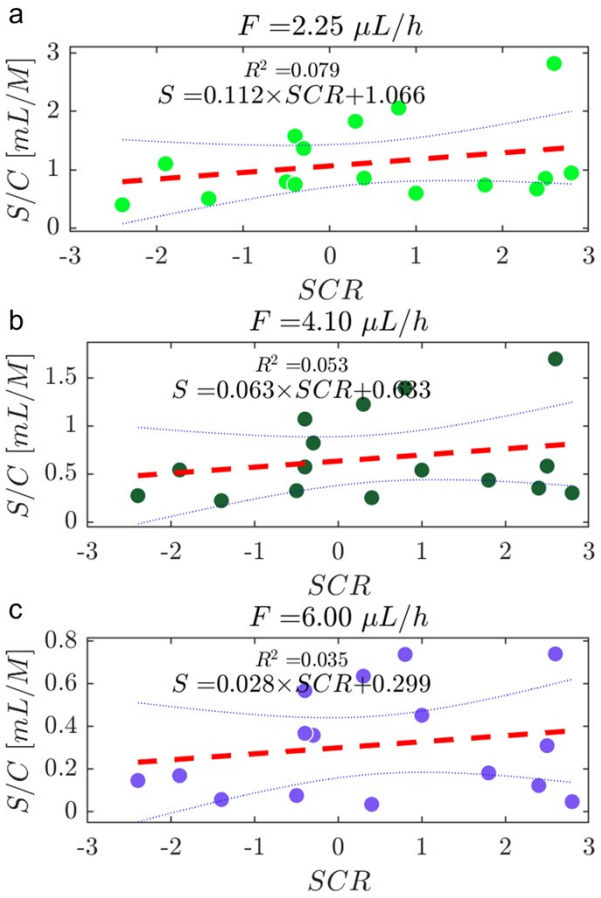

Figure S11. Relative RHEOLEX over SCR for flow rates of (a) 2.25 (b) 4.1, and (c) 6 µL h^{-1}. Dotted lines in the figures show the 95% confidence interval.

Table S1. Data of the RHEOLEX, flow rates and concentrations used for linear model of bull fertility. The height here refers to the injection height of the inlet reservoir in Figure S1.

Concentration [M/mL]	Height 20mm (N=3)	Height 40 mm (N=3)	Height 60 mm (N=3)
SCR=2.80 conc=1.87			
Flow rate [μL/h]	1.87 (0.13)	4.52 (0.12)	5.30 (0.14)
Signal [a.u.]	2.25 (0.50)	0.30 (0.17)	0.23 (0.06)
SCR=2.50 conc=3.04			
Flow rate [μL/h]	2.54 (0.06)	5.25 (0.04)	5.84 (0.65)
Signal [a.u.]	2.48 (0.48)	1.22 (0.68)	1.01 (0.29)
SCR=-1.90 conc=2.46			
Flow rate [μL/h]	2.34 (0.23)	4.31 (0.40)	5.51 (0.32)
Signal [a.u.]	5.84 (1.45)	1.94 (0.15)	1.31 (0.45)
SCR=-2.40 conc=1.50			
Flow rate [μL/h]	1.88 (0.08)	2.31 (0.23)	6.03 (0.44)
Signal [a.u.]	0.85 (0.07)	0.44 (0.15)	0.22 (0.13)
SCR=0.40 conc=1.62			
Flow rate [μL/h]	1.99 (0.10)	2.64 (0.16)	4.20 (0.11)
Signal [a.u.]	2.06 (0.82)	0.79 (0.36)	0.40 (0.25)
SCR=0.80 conc=0.74			
Flow rate [μL/h]	3.00 (0.45)	5.70 (0.67)	6.72 (0.31)
Signal [a.u.]	1.43 (0.17)	0.50 (0.23)	0.45 (0.31)
SCR=-0.50 conc=5.01			
Flow rate [μL/h]	1.89 (0.26)	4.77 (0.11)	5.58 (0.27)
Signal [a.u.]	4.28 (0.70)	1.18 (0.45)	0.50 (0.34)
SCR=1.00 conc=2.74			
Flow rate [μL/h]	2.30 (0.26)	3.74 (0.24)	8.23 (0.73)
Signal [a.u.]	2.02 (0.97)	1.19 (0.24)	0.96 (0.10)
SCR=1.80 conc=3.40			
Flow rate [μL/h]	2.30 (0.26)	4.28 (0.11)	5.60 (0.27)
Signal [a.u.]	2.66 (1.54)	1.21 (0.59)	0.81 (0.20)
SCR=-0.40 conc=1.30			
Flow rate [μL/h]	2.79 (0.23)	3.80 (0.23)	6.00 (0.53)
Signal [a.u.]	2.13 (0.63)	1.25 (0.52)	0.76 (0.24)
SCR=0.30 conc=0.92			
Flow rate [μL/h]	3.05 (0.64)	4.46 (0.15)	8.13 (0.43)
Signal [a.u.]	2.24 (0.32)	0.60 (0.12)	0.23 (0.19)
SCR=-0.30 conc=1.52			
Flow rate [μL/h]	2.92 (0.42)	4.02 (0.18)	5.09 (0.04)
Signal [a.u.]	1.83 (0.21)	1.22 (0.13)	0.86 (0.30)
SCR=-1.40 conc=1.27			
Flow rate [μL/h]	2.56 (0.30)	3.74 (0.26)	3.99 (0.12)
Signal [a.u.]	0.58 (0.23)	0.33 (0.08)	0.30 (0.11)
SCR=2.40 conc=2.01			
Flow rate [μL/h]	2.10 (0.15)	4.01 (0.08)	7.15 (0.19)
Signal [a.u.]	1.61 (0.69)	0.61 (0.33)	0.11 (0.11)
SCR=2.60 conc=1.10			
Flow rate [μL/h]	1.87 (0.29)	4.79 (0.60)	6.55 (0.91)
Signal [a.u.]	3.45 (0.90)	1.35 (0.54)	0.63 (0.28)
SCR=-0.40 conc=1.97			
Flow rate [μL/h]	1.04 (0.1)	3.33 (0.06)	6.07 (0.39)
Signal [a.u.]	1.63 (0.25)	1.27 (0.29)	0.71 (0.36)

11- CASA parameters of the 16 bulls and progressive motility

Table S2. CASA parameters of the different bulls along with their SCR values.

SCR	Concentration [M/mL]	VCL [µm/s]	VSL [µm/s]	VAP [µm/s]	BCF [Hz]	ALH [µm]
-2.4	1.50(0.53)	122.51 (48.11)	40.41 (27.64)	49.08 (24.11)	21.73 (4.92)	1.73 (0.71)
-1.9	4.91(0.70)	161.31 (74.06)	50.12 (31.38)	61.69 (29.19)	19.52 (4.58)	2.44 (1.14)
-1.4	1.27(0.18)	88.23 (45.92)	28.21 (22.78)	34.70 (21.23)	17.74 (5.01)	1.39 (0.65)
-0.5	5.01(0.37)	125.32 (61.26)	47.09 (28.25)	54.41 (24.58)	20.35 (5.54)	1.77 (0.86)
-0.4	1.29(0.21)	116.03 (48.34)	50.80 (25.42)	57.35 (24.59)	20.37 (5.50)	1.67 (0.64)
-0.4	1.97(0.12)	134.82 (46.54)	50.38 (24.80)	57.39 (21.27)	20.13 (4.38)	1.94 (0.67)
-0.3	1.52(0.38)	94.51 (36.17)	40.94 (26.45)	52.04 (21.92)	15.36 (3.32)	1.72 (0.64)
0.3	0.92(0.06)	105.42 (49.82)	42.49 (25.53)	57.11 (25.67)	15.99 (3.67)	1.86 (0.85)
0.4	1.62(0.57)	101.16 (41.19)	47.74 (29.70)	56.16 (22.05)	16.35 (3.51)	1.74 (0.70)
0.8	0.74(0.00)	142.44 (50.56)	48.09 (26.75)	67.25 (21.54)	16.27 (2.93)	2.58 (0.91)
1	2.75(1.03)	77.01 (41.60)	34.85 (28.96)	46.79 (27.36)	15.18 (4.38)	1.47 (0.80)
1.8	3.40(0.09)	144.26 (42.92)	58.13 (24.38)	63.61 (20.11)	20.35 (4.39)	2.04 (0.67)
2.4	2.01(0.52)	91.54 (47.66)	35.19 (24.57)	48.04 (24.90)	15.03 (3.56)	1.74 (0.94)
2.5	3.04(0.34)	127.53 (56.62)	48.35 (27.14)	56.45 (24.41)	19.84 (4.56)	1.88 (0.82)
2.6	1.11(0.22)	144.96 (52.73)	50.23 (24.78)	57.77 (19.43)	18.53 (3.92)	2.16 (0.73)
2.8	1.87(0.22)	81.35 (50.98)	48.87 (21.70)	47.33 (25.49)	10.33 (2.45)	2.76 (0.54)

At least 50 sperm were counted for evaluation of CASA parameters. None of the parameters presented here showed any relationship with fertility levels or SCR. Quantities are mean (standard deviation).

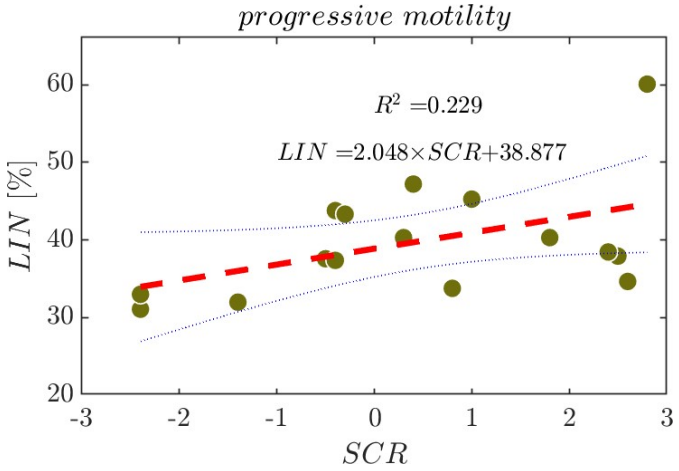

Figure S12. Progressive motility of bull samples with respect to SCR. Although this shows a positive trend the slope is not statistically significant (p value > 0.05). LIN is a CASA parameter calculated by the ratio of VSL to VCL commonly known as progressive motility.

12- Appendix

Here the algorithm used for the calculation of the signal from the videos is described. First, the location of the corner is detected and then the picture is rotated around the corresponding point to make the image horizontal. Some of the movies are captured while the chip is not placed perfectly horizontally aligned with the camera under the microscope and this way the results are consistent.

The mean value of the intensity of a window of 1000×1000 pixels around the corner of the images is scaled to 2000. Then windows with 434×620 pixels were cut from the images and a mask was used to cover the areas outside of the corner to remove unwanted signals from the motion of the egg-yolk extender particles. The left corner of this window is placed at X = 0 and Y = –310 in the coordinate system attached to the midline on the left edge.

After each of these cut images is subtracted from its preceding image, intensities under 1000 are suppressed to zero and the maximum intensity of this image is rescaled to its mean value to suppress steeped gradients of intensity in each image. At the end, a median filter is used to remove noise and this process is repeated for each of the 50 images resulting in 49 Signal images. Intensity averages of these images are used for the value of the signal for each subtracted image, which are then time-averaged for the signal of that particular time point.

13- Movie Captions

Movies S1: The signal over time for the case shown in Figure 3a ($C = 2.04$ M mL^{-1} and $F = 3.95$ $\mu L\ h^{-1}$)

Movie S2: The signal over time for $C = 4.2$ M mL^{-1} and $F = 1.0$ $\mu L\ h^{-1}$. The signal increases as the sperm number within the channel increases and the resolution of the sperm number decreases but the signal is not saturated.

Movie S3: At a corner angle of 50° sperm are able to deviate out of the probe.

14- References

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