

First-line Evaluation of Sperm Parameters in Mice (*Mus musculus*)

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Abstract

Infertility has become a major public health problem, with a male factor involved in about half the cases. Mice are the most widely used animal model in reproductive biology research laboratories, but changes in sperm parameters in mice can be subtle and, in the absence of official guidelines, it is important that analyses are carried out in a strict and reproducible manner. This protocol successively details the different steps required to obtain spermatozoa under good conditions, the measurement of sperm motility using a Computer Assisted Sperm Analysis System (CASA) device, the calculation of sperm concentration in the epididymides using a sperm counting cell, and the examination of sperm morphology. The combination of these assays provides an overview of the basic sperm parameters in mice. This is both a diagnostic and a decision-making tool for researchers to orient their scientific strategy according to the observed abnormalities.

Keywords: Sperm, Mouse, CASA, Sperm morphology, Sperm motility, Sperm count

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Background

Mice are the most widely used model organism in laboratories around the world because of their relatively simple breeding conditions, short reproduction cycles, and physiology close to humans. Several fields of science use mice as model organisms, and the advent of genetically manipulated mouse breeding has further expanded the possibilities of this model (Olson et al., 2018; Ito et al., 2018; Gill et al., 2020). For example, CRISPR technology now allows the efficient generation of mouse knock-out models to functionally validate newly identified genetic mutations in a wide range of diseases (Abbasi et al., 2017; Hochheiser et al., 2018; Zhao et al., 2020), including infertility (Beurois et al., 2020; Touré et al., 2021).

Medically assisted reproduction techniques are implemented in the murine model (Stein et al., 2010), establishing it as a model of choice for reproductive biologists (Giritharan et al., 2010), for whom the analysis of sperm parameters became essential a long time ago (Osterloh et al., 1983; Tessler et al., 1985). These techniques have since been adopted by a large portion of scientists who use them for different purposes, such as the evaluation of the impact of diseases and/or toxic substances on reproduction (Sampannang et al., 2020; Abdollahi et al., 2021) and the curative and/or protective effects of molecules against induced deleterious effects (Feyli et al., 2017; Moghadam et al., 2021; Shahedi et al., 2021). Other noteworthy examples include the analysis of nutrition influence on sperm parameters (Zhao et al., 2017; Gómez-Elías et al., 2019) and their heritability (Crisóstomo et al., 2021), or the potential of cryobiology for reproduction (Sztejn et al., 2018).

However, this model suffers from limitations in its transfer to humans. One of the drawbacks of the mouse model is that mice have a much more efficient spermatogenesis and much higher fertility than humans—already the primate with the worst known sperm parameters (Martinez and Garcia, 2020). This implies that even subtle defects in mouse sperm parameters may eventually translate into a much more deleterious impact in humans (Martinez et al., 2022). That is why descriptions of animal reproductive phenotypes are becoming increasingly sophisticated and standardized, with each specialty creating its own phenotyping framework (Houston et al., 2021). Here, we propose a rapid, accessible, and reproducible protocol for the first-line assessment of sperm parameters in mice, which provides the researcher with a first diagnosis to guide their scientific strategy and the framework of their future experiments.

This protocol sequentially describes the surgical steps of sperm recovery, sperm motility measurement using a Computer Assisted Sperm Analysis System (CASA) device, sperm concentration measurement, and the examination of sperm morphology.

Materials and Reagents

1. Petri dish polystyrene (Falcon, catalog number: 351008)
2. Box with instruments for minor surgery (LD Medical, catalog number: 01748)
3. Needles, Microlance™ 3, 18 G (Beckton Dickinson, catalog number: 304622)
4. Microtube safety lock 2 mL Clearline (Dutscher, catalog number: 2519649)
5. SureOne specialty tips (Fisherbrand, catalog number: 02-707-464)
6. Counting chamber Leja 100 µm (Leja Products B.V., Gynemed, catalog number: SC100-01-02-A)
7. Thoma Cell Counting chamber (VWR, catalog number: HECH40447702)
8. Tubes 15 mL (Falcon, catalog number: 352096)
9. SuperFrost Plus™ adhesion slides (Eprexia, catalog number: J7800AMNZ)
10. Soda lime glass staining troughs with lid (Brand, catalog number: 472200)
11. EUKITT® classic 100 mL flask (Dutscher, catalog number: 124103)
12. Mice (Charles River Laboratories)
13. M2 medium (Sigma-Aldrich, catalog number: M7167-50ML)
14. Ethanol 96% (Carlo Erba, Labelians, catalog number: CE529141)
15. Sterile water (Versylene, Fresenius)
16. Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: D8537-500ML)
17. Papanicolaou's solution 1a Harris' hematoxylin solution (Sigma-Aldrich, catalog number: 1092530500)

18. Shorr staining solution stain for histology (Sigma-Aldrich, catalog number: 1092750500)
19. Ammonia solution 25% (Sigma-Aldrich, catalog number: 105432)
20. Immersion oil for microscopy type N (Nikon, MXA22166)
21. 75% ethanol (see Recipes)
22. 50% ethanol (see Recipes)
23. Ethanol ammoniacal (see Recipes)

Equipment

1. Surgical scissors
2. Incubator binder model CB56 (Binder, catalog number: 9640-0006)
3. Binocular loupe (Nikon, catalog number: SM2800N)
4. IKA dry block heater 3 (IKA, catalog number: 0004025300)
5. Computer Assisted Sperm Analysis System (CASA) CEROS II (Hamilton Thorne Research, IMV Technologies, catalog number: 024905) with its heated blade holder
6. Centrifuge Sorvall ST 16R (ThermoFisher, catalog number: 10688725)
7. Nikon Eclipse 80i microscope equipped with a Nikon DS-Ri1 camera with NIS-Elements software and 40× and 100× objectives

Procedure

Notes:

1. *The first two steps of this protocol (sperm recovery and motility analysis) are performed under time and temperature constraints. Sperm motility is a physiological phenomenon that evolves over time and is impacted by the temperature of the environment. To obtain reproducible motility data, the time between sperm extraction and motility measurement has to be the same for all individuals in the study (optimal time would be between 15 and 20 min), and a temperature of 37 °C has to be maintained throughout. As concentration and morphology measurements are not impacted by these factors, they can then be carried out comfortably afterwards, without restrictions.*
2. *When handling sperm, always use a cut or wide-opening pipette tip (see Figure 1). The use of normal tips induces deleterious effects on the motility and morphology of spermatozoa.*



Figure 1. Use wide-opening pipette tips

A. Before beginning the procedure

1. Prepare Petri dishes (two per individual, one per epididymis) with 1 mL of M2 medium and one tube with 5 mL of M2 medium. Heat them to 37 °C for at least 2 h before starting the experiment.
2. Prepare the dissection field.

- Preheat the heating block to 37 °C and place 2 mL microtubes in it (see Figure 2).

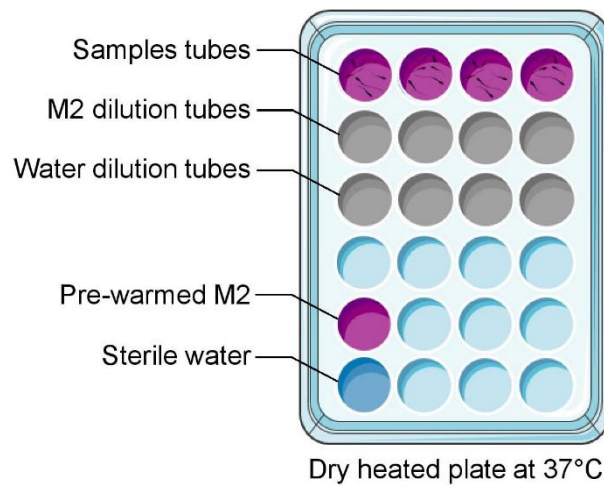


Figure 2. Suggested organization of the heating block

- Connect the heated blade holder of the CASA.
- Turn on the computer, start the CASA software (Figure 3), and enter the sample numbers if appropriate.
Note: The sperm analysis software must have the animal analysis module and be configured according to its user manual. We provide the parameters to be used for mice sperm analysis in Table 1, but we will not go over all the basic configuration elements. For this, please refer to the CEROS II manual.

Table 1. Settings for CASA analysis

Parameters	Value	Parameters	Value
acquisition rate	60 Hz	low static–intensity gate	0.5
number of frames	45	high static–intensity gate	1.3
minimum contrast	50	minimum elongation gate	0
minimum cell size	5	maximum elongation gate	87
low static-size gate	0.3	magnification factor	0.7
high static-size gate	1.95		
motile sperm	VAP >1	progressive motility	VAP >30 + STR >70

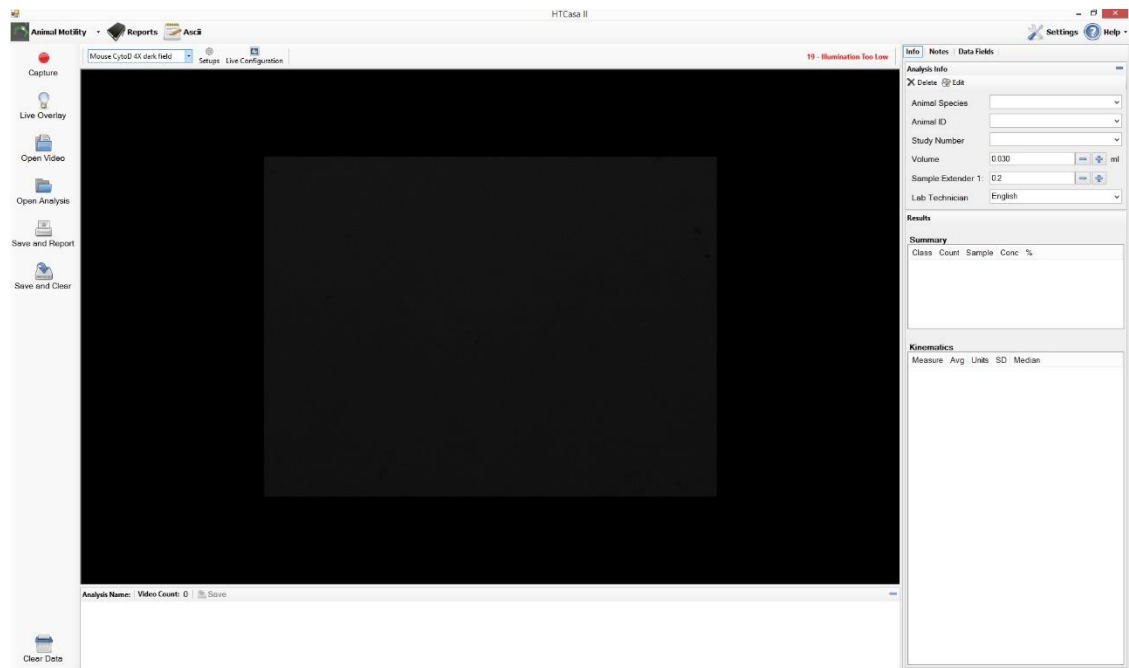


Figure 3. Screenshot of the software window

B. Sperm recovery

1. Sacrifice the animal according to the procedures established in your country and validated by your local ethics committees.
Note: To obtain reproducible and exploitable data, all animals in the same study should be sacrificed at the same age (we recommend between 10 and 12 weeks for an evaluation of the reproductive function in the mature male).
2. Immediately put the animal on its back on a sterile surgical field and disinfect the abdominal and genital area with ethanol.
Note: Simply spray or apply ethanol with a sterile compress.
3. Use surgical scissors to make a horizontal skin incision from one lumbar region to the other (Figure 4A), and a large vertical incision through the hypogastric region to the pelvis region (Figure 4B).
4. Expose the organs by incising vertically through the abdominal muscle wall (Figure 4C).
Note: A quick check will allow you to spot unexpected flagrant anomalies such as a situs invertus.
5. Locate (Figure 4D) and pull upward with surgical clamps the fat deposits located on each side of the pelvic fossae to elevate and expose the testicles (Figure 4E).
Note: Be careful not to puncture the bladder.



Figure 4. Sequence of surgical procedures for exposure of reproductive organs

6. Locate the different structures of the reproductive tract from the testis (Figure 5A). Start with the head of the epididymis visible on the testicle and then proceed to the body, tail, and then the efferent canal.
7. Isolate the tail of the epididymis by incising at one millimeter past the tail-canal junction distally (Figure 5B) and at the body-tail junction proximally (Figure 5C), and place it immediately in the pre-warmed M2 medium in Petri dish.

Note: Optimal time for steps 1–7 is less than 10 min.



Figure 5. Sequence of surgical procedures for epididymal tail isolation with indication of testis (asterisk), epididymal head (cross), and tail (arrow)

8. Dissect the epididymis with two needles under a binocular loupe with a preheated plate at 37 °C (Figure 6).

Note: It is important to completely drain the tail of the epididymis to obtain the correct concentration of spermatozoa. The dissection must be done very quickly, which can be challenging for a beginner. In this case, always take the same amount of time to dilate the epididymes of the different individuals of the same study. Optimal time for step 8 is less than 10 min (for both epididymis).

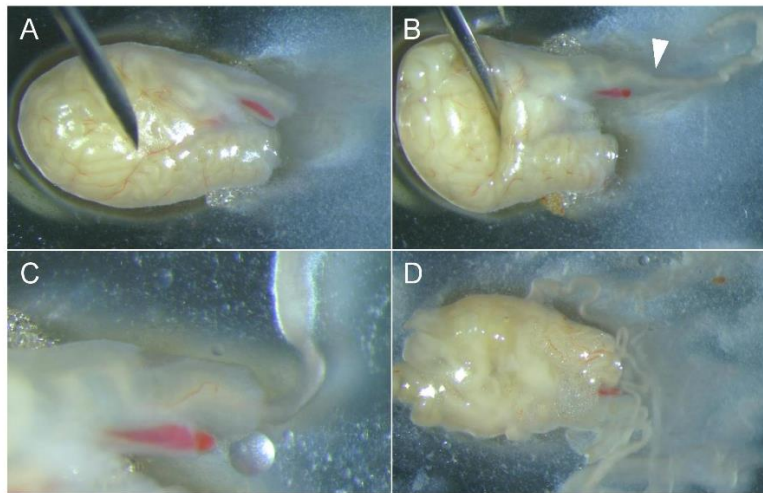


Figure 6. Place the tail of the epididymis so that the incisions are clearly visible.

(A) Apply pressure to the tail of the epididymis with a needle to remove the sperm through the vas deferens. (B) The spermatozoa will come out as a jelly that will disintegrate in the medium. (C) Empty the epididymis completely by applying repeated pressure. (D) It may be necessary to pierce the organ in several places to empty it completely.

9. Place the Petri dishes in an incubator at 37 °C for 10 min to allow the sperm to gain motility.
Note: These ten minutes can be used to adjust the CASA settings and fill in the sample identification in the software, if not already done beforehand.

C. Sperm motility assessment

1. Transfer the sperm in M2 medium from the Petri dish to the 2 mL microtube preheated in a 37 °C heating block.
Note: You can pool the two epididymides of the same individual or treat them separately according to your needs.
2. Take 20 μ L of suspended semen, place it in a second Eppendorf, then dilute it with preheated M2 medium.
Note: The dilution performed depends on the mice strain used. Perform an experiment beforehand on a control individual to identify the dilution to be used for the rest of the study. For example, we use a standard 1:50 dilution for our Black 6 mice but a 1:100 dilution for our OF1 mice.
3. Homogenize and then transfer the sample to a Leja slide. Put the slide in the heated blade holder under the microscope.
Note: Wait a few moments for the flow to stabilize in the chamber to prevent the sperm trajectories from being affected by any residual Brownian motion.
4. Confirm that the sperm density is in the acceptable range of the software and check that the sample identification is correct.
Note: The software acceptability cut-off is relatively high. We advise to use a concentration that does not lead to a systematic crossing of the trajectories to allow an easier analysis afterwards.
5. Acquire with the software as much field as you may need.
Note: For a statistically robust analysis, we recommend acquiring a minimum of 200 motile spermatozoa. Use a “forward walk” reading during the acquisition to avoid taking the same field several times (Figure 7).

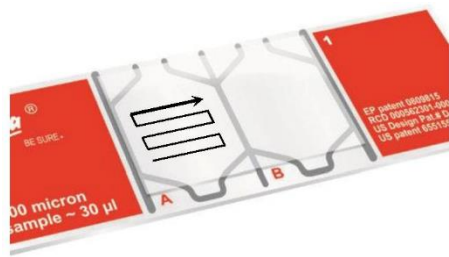


Figure 7. “Forward walk” reading path on a Leja slide

6. Process all samples successively in the same way.

Note: The purpose here is to acquire the data (the videos) to being able to interpret them later. Do not waste time interpreting now and focus on acquiring all the samples sequentially.

D. Sperm concentration assessment

Note: We describe here the method used for a Thoma chamber; however, you can use any other type of counting chamber (e.g., Mallassez or Makler) as long as you follow the associated instructions.

1. Take 10 μL of suspended semen, place it in a third Eppendorf, and add 90 μL of sterile H_2O to immobilize the spermatozoa.

Note: You can use another dilution, but you will have to take it into account when calculating the final concentration.

2. Homogenize and transfer 10 μL to the Thoma counting chamber.
3. Count the number of sperm present on two lines to obtain the sperm concentration expressed in millions per milliliter (Figure 8).

Note: Count the sperm heads; single flagella are not counted or counted separately. This is also an opportunity to calculate the concentration of any round cells in the ejaculate (germline or immune cells). If you have pooled epididymides, do not forget to multiply by two. You can count more lines and average the results for more accuracy.

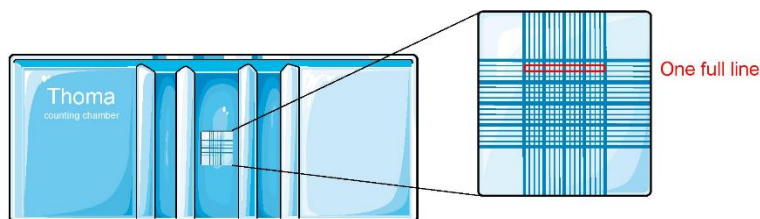


Figure 8. Count two full lines of a 1:10 diluted sample to obtain the cell concentration in millions per milliliter

E. Sperm morphology assessment

1. Transfer the remaining samples to 15 mL tubes. Add 3 mL of $1\times$ PBS. Homogenize by gentle pipetting.
2. Centrifuge at $400 \times g$ for 5 min. Discard the supernatant.
3. Add another 3 mL of $1\times$ PBS to the pellet.
4. Homogenize by gentle pipetting.
5. Centrifuge at $400 \times g$ for 5 min. Discard the supernatant.
6. Add another 200 μL of $1\times$ PBS to the pellet.
7. Homogenize by gentle pipetting.
8. Put 10 μL (this volume can be adjusted according to the desired concentration of cells on the slide) at the

base of a clean slide and make a smear with a second slide.

Note: The drop must be "pulled" and not "pushed" by the second blade, which would break the sperm flagella (see Figure 9).

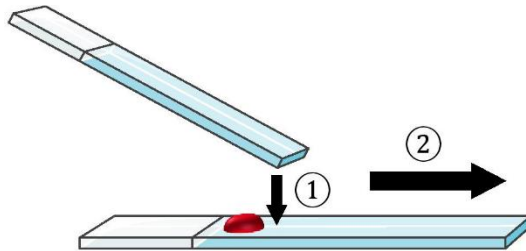


Figure 9. The smearing blade should be placed in front of the drop and pull it forward.
It must not push or cut through the drop.

9. Let dry at room temperature.
10. Immerse in a 75% ethanol bath for at least 1 h.
11. Let dry at room temperature.
12. Perform a Harris-Schorr staining by carrying out the following sequence of baths (one immersion corresponds to a duration of approximately 1 s):
 - a. Tap water—15 immersions
 - b. Hematoxylin—2 min
 - c. Tap water—15 immersions
 - d. Ethanol ammoniacal—10 immersions
 - e. Tap water—15 immersions
 - f. Ethanol 50%—5 min
 - g. Shorr—5 min
 - h. Ethanol 50%—5 min
 - i. Ethanol 75%—5 min
 - j. Ethanol 95%—5 min
13. Allow the stained slides to dry completely.
Note: Any alcohol residue will interfere with the interpretation.
14. Mount the slides with Eukitt and a 24 mm × 60 mm cover slide.
Note: Avoid trapping air bubbles and remove them by applying gentle pressure to the cover slide if necessary.
15. Let the mounted smear dry horizontally on absorbent paper for 24 h before interpretation.

Data analysis

A. Concentration analysis

1. Data can be presented as cell concentration in million cells per milliliter, number of cells per epididymis, or total numeration of cells per individual.

B. Motility analysis

1. Review each acquired video (Figure 10) to ensure that the selected sperm paths are correct, i.e., eliminate possible experiment artifacts (air bubble, residue, aggregates) and intersecting sperm paths.
Note: For more details, please refer to the CEROS II user manual.

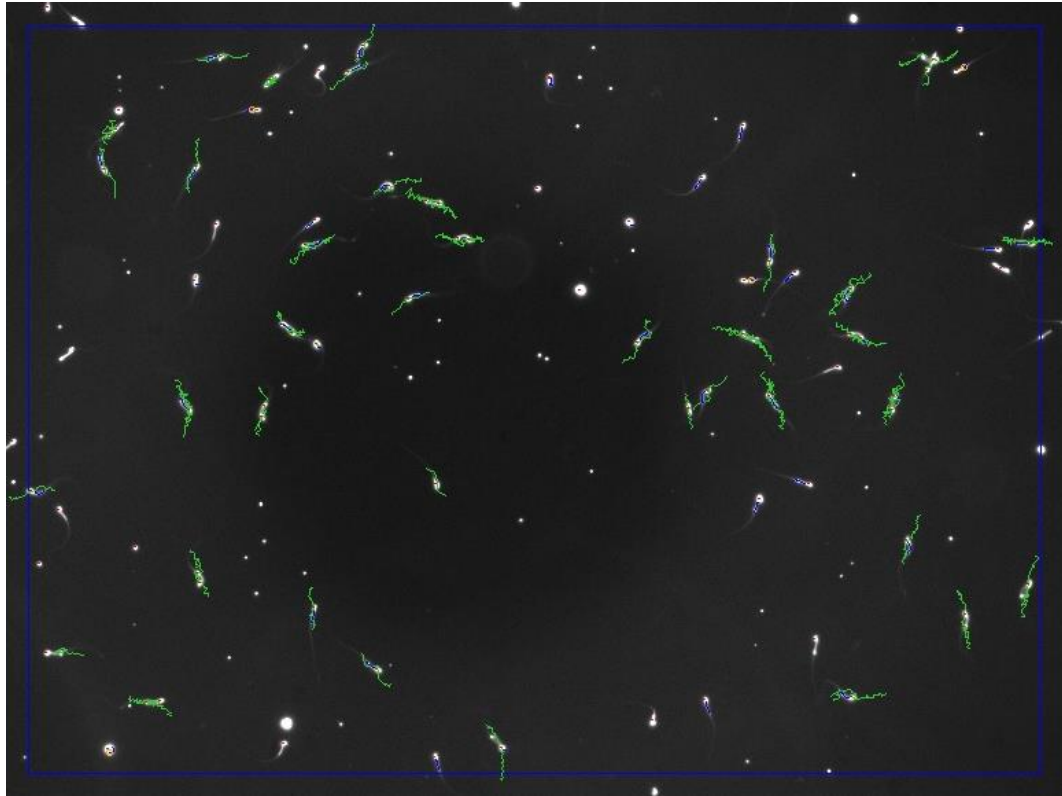


Figure 10. Screenshot of pattern analysis from the software.

Motile sperm pathways are highlighted in green.

2. The software will provide you with all the information about sperm motility (Figure 11): a first part with the percentages of motile and progressive motile sperm, a second part (if necessary) with the main parameters—the curvilinear velocity (VCL) or average path velocity (VAP), straight-line velocity (VSL), and amplitude lateral of the head (ALH)—and then if necessary the subsequent findings (e.g., the percentage of capacitated sperm).

Note: Be careful with how you handle the results given by the software, as they are in the form of mean/median \pm standard error/deviation. Depending on the analysis you want to do, it could be wiser, statistically speaking, to start directly from the raw data of each trajectory extractable from the software.

Results

Summary

Class	Count	Sample (M)	Conc (M/ml)	%
Total	718	0.02	0.60	100
Static	334	0.01	0.28	46.5
Motile	384	0.01	0.32	53.5
Progressive	349	0.01	0.29	48.6
Slow	16	0.00	0.01	2.2

Kinematics

Measure	Avg	Units	SD	Median
Total				
DAP	37.39	µm	39.24	76.54
DSL	33.47	µm	35.97	68.76
DCL	59.98	µm	62.82	115.38
VAP	74.6	µm/sec	73.59	142.13
VSL	66.54	µm/sec	66.79	129.48
VCL	120.23	µm/sec	118.81	222.62
STR	47.72	%	46.3	92.13
LIN	30.35	%	30.39	57.36
ALH	5.66	µm	6.05	10
BCF	15.77	Hz	29.57	26.93
WOB	33.85	%	32.76	63.63
Static				
DAP	0.01	µm	0.12	1.06
DSL	0.01	µm	0.12	1.06
DCL	0.12	µm	0.76	1.66
VAP	0.02	µm/sec	0.37	3.35
VSL	0.02	µm/sec	0.37	3.35
VCL	0.02	µm/sec	0.37	3.35
STR	0.3	%	5.47	50
LIN	0.3	%	5.47	50
ALH	0	µm	0.05	0.43
BCF	1.82	Hz	33.26	303.92
WOB	0.3	%	5.47	50

Figure 11. Screenshot of results provided by the software post-analysis

C. Morphology analysis

- Analyze the slides using a brightfield light microscope with the 100× objective and immersion oil.
- The head is stained pale blue in the acrosome region and dark blue in the post-acrosomal region. The intermediate piece is stained red and the flagellum blue or red. The cytoplasmic residues are colored red–orange.
- Differentiate spermatozoa according to their typical/atypical morphology (Figure 12) on a minimum of 100 cells (200 for more precise analysis).

Note: It is possible to limit the analysis to the typical/atypical character of the spermatozoa, but it is interesting to carry out an in-depth analysis by separating the abnormalities of the head and the flagellum, or even going into the details of the abnormalities. For this purpose, it is necessary to distinguish abnormalities of the head [elongated, thinned, microcephalic, macrocephalic, multiple, with an abnormal or absent acrosome, or with an abnormal base (post-acrosomal region)], the intermediate piece (which may be small, angular, or with cytoplasmic remains), and the flagellum (which may be absent, shortened, of irregular caliber, coiled, and/or multiple).

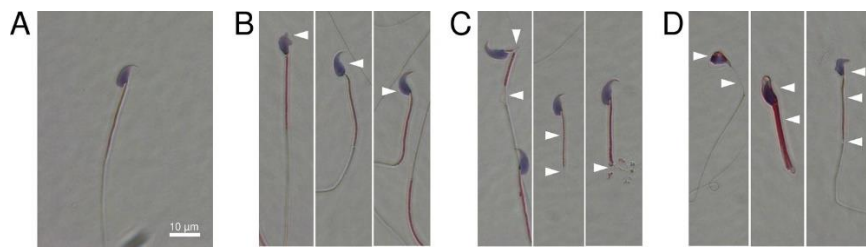


Figure 12. Light microscopy pictures of Harris-Schorr stained spermatozoa (from Martinez et al., 2022). (A) Typical morphology. (B) Head abnormalities. (C) Flagellum abnormalities. (D) Head and flagellum abnormalities. Defects are pointed by white arrows.

Notes

In an effort to refine and reduce the number of animals used in the studies, always consider additional analyses whenever possible: fix the testicles to make histological sections or the remaining spermatozoa pellets to make immunofluorescence slides, freeze for proteomics, extract DNA from other organs, and run toxicology analyses. Whatever your field of study, try to plan for simultaneous manipulations.

Recipes

1. **75% ethanol**
100 mL of 96% ethanol + 31 mL of sterile water
2. **50% ethanol**
100 mL of 96% ethanol + 98 mL of sterile water
3. **Ethanol ammoniacal**
95 mL of 75% ethanol + 5 mL of ammonia solution at 25%

Acknowledgments

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Competing interests

The authors declare that no competing interests exist.

Ethics

All animal procedures presented in the original paper were conducted according to a protocol approved by the local Ethics Committee (ComEth Grenoble No. 318), by the French government (ministry agreement number #7128

UHTA-U1209-CA), and by the Direction Générale de la Santé (DGS) for the State of Geneva.

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