



Automated sperm tracking using deep learning for high-resolution analysis of motility

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In brief

Accurate tracking of sperm cells is critical in the assessment of sperm motility. This work presents an open-source workflow for tracking sperm of any species that can be readily implemented with any phase-contrast microscope.

Abstract

Sperm motility is a key determinant of cell function, and it is critical for male fertility. In the analysis of human semen, motility is routinely assessed using manual evaluation or computer-assisted sperm analysis (CASA). However, these approaches provide limited access to single-cell dynamics, use proprietary software, and rely on population- and time-averaged descriptors of sperm motion that can obscure both the intrinsic heterogeneity and the dynamic behaviors within individual trajectories. Here, we present an open-source, modular framework for automated sperm detection and tracking that enables trajectory-resolved quantification across species. The pipeline combines deep learning-based sperm head segmentation using species-specific Cellpose models with automated tracking via Trackpy, enabling reconstruction of individual sperm trajectories from time-lapse microscopy. This workflow was applied across four species: human, mouse, rat, and bovine. In human sperm, it was further tested under both basal and hyperactivation-promoting conditions. Detection performance was consistently high across species, with F1 scores, a metric that measures model accuracy, between 0.96 and 0.98 on independent validation datasets. From the obtained trajectories, CASA-like parameters, such as curvilinear velocity, straight-line velocity, and amplitude of lateral head displacement, were computed, showing strong agreement in human sperm with conventional CASA. An automated Python-based software for sperm tracking was designed to facilitate access to the technology. Overall, this framework provides a transparent and flexible alternative to CASA, enabling high-resolution, single-cell analysis of heterogeneous sperm motility and offering new opportunities to study sperm function.

Keywords sperm motility, computer assisted semen analysis, machine learning

Sperm motility characterization is a cornerstone of reproductive studies, providing a functional fingerprint of cell function. Motility serves not only as a diagnostic indicator of reproductive potential but also as a window into the underlying biophysical and metabolic processes governing cell activity. In human clinical practice, semen analysis remains the primary diagnostic tool for assessing male infertility, with evaluation typically based on sperm concentration, motility, and morphology following standardized protocols established by the World Health Organization (World Health Organization, 2021). Among these parameters, sperm motility is

critical, as sperm must actively navigate the female reproductive tract to reach and fertilize the oocyte (Gaffney et al., 2011; Suarez & Pacey, 2006). In practice, sperm motility is evaluated either manually by trained technicians or via computer-assisted sperm analysis (CASA) systems (Mortimer, 1988). These automated approaches provide standardized measurements of kinematic parameters such as velocity, linearity, and beat frequency. However, they also possess notable limitations (Amann & Waberski, 2014; Mortimer et al., 2015; Gallagher et al., 2018). In particular, CASA systems are often costly, which can restrict accessibility for clinical and

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research use in resource-limited settings. Many platforms are proprietary and their “black box” algorithms limit flexibility for methodology development. In addition, commonly reported CASA parameters are typically time-averaged descriptors of sperm motion, which may fail to capture the dynamic and heterogeneous behaviors present within sperm populations (Brisard et al., 2025). Recent studies have demonstrated that trajectory-based analyses and machine learning approaches can provide complementary information for identifying kinematic heterogeneity and sperm subpopulations beyond conventional CASA descriptors (Aragón-Martínez, 2026; Rodríguez-Martínez et al., 2023).

Sperm populations are far from uniform regarding both motility patterns and physiological responses (Suarez, 2008). Individual sperm may display diverse trajectories, transitions between distinct motility states, and variable responses to capacitating conditions. During capacitation, a final sperm maturation step that occurs in the female reproductive tract, sperm undergo a series of molecular and motility changes required for fertilization (Novero et al., 2025; Visconti, 2009; Visconti et al., 1995). One of the key functional outcomes of capacitation is the development of hyperactivation, a high-amplitude, asymmetric flagellar beating pattern characterized by increased curvilinear velocity and reduced linearity, which is essential for successful navigation of the female tract and penetration of the oocyte vestments (Gaffney et al., 2011; Suarez & Pacey, 2006). This motility state is regulated by intracellular signaling pathways involving cyclic adenosine monophosphate (cAMP), and it can be experimentally induced or mimicked using cAMP analogs that trigger capacitation-like signaling events (Buffone et al., 2014; Garbers et al., 1971; Leclerc et al., 1996; Visconti et al., 2024). As a result, motility evolves dynamically and heterogeneously, and a more detailed characterization of sperm trajectories and population dynamics may provide deeper insights into sperm function.

Recent advances in computer analysis and machine learning have greatly improved the ability to detect and track individual particles in complex imaging datasets (Ershov et al., 2022; Volpe et al., 2026). In parallel, open-source image analysis platforms have become central tools for biological image processing, enabling reproducible and extensible workflows (Chiu et al., 2022; Schindelin et al., 2012). Deep learning-based segmentation methods offer improved robustness in challenging imaging conditions, including high cell density, debris, and variable contrast (Stringer et al., 2021; Weng & Zhu, 2021). Despite these advances, there is still a lack of accessible, transparent, and flexible tools that enable robust sperm trajectory analysis while capturing the dynamic heterogeneity of sperm populations.

Here, we present an open-source platform for automated detection and tracking sperm. The software is openly available on GitHub (<https://github.com/zindrok/automated-sperm-tracking>). Our approach combines deep learning-based sperm detection with trajectory extraction, providing a modular and reproducible workflow for studying sperm movement. The combination of automated trajectory extraction and quantitative analysis tools creates new opportunities to investigate sperm motility at the level of individual trajectories and population dynamics. Other open-source CASA platforms provide access to sperm coordinates and trajectory reconstruction (Alquézar-Baeta et al., 2019; Giaretta et al., 2017; Rodríguez-Martínez et al., 2023). Our framework combines deep learning-based sperm detection with

automated tracking, species-specific pretrained models, and an open-source workflow that facilitates customizable downstream analyses. This approach enables the study of dynamic behaviors that are often obscured by population- and time-averaged metrics, which mask both cell-to-cell heterogeneity and intratrajectory dynamic variability. This framework enables the extraction of trajectory-associated metrics, allowing the direct characterization of motility patterns and population heterogeneity. By prioritizing flexibility, this platform is designed to support research applications and the development of improved analytical tools for sperm motility assessment, with potential future applications in guiding clinical decision-making and optimizing treatment strategies for male infertility.

Materials and methods

Ethical aspects

All experimental procedures involving human and animal sperm were conducted in accordance with institutional and international ethical guidelines. Protocols involving human sperm were reviewed and approved by the Ethics Committee of the Instituto de Biología y Medicina Experimental (IBYME), Buenos Aires (CE 12769) and the Institutional Review Board of Colorado State University (CSU), Colorado (IRB 7208). Written informed consent was obtained from all donors. Protocols involving mice, rats, and bovine were reviewed and approved by the Institutional Animal Care and Use Committee of CSU (IACUC 4759) and IBYME (CICUAL 08/2023), as applicable, and were conducted in accordance with national and international regulations for the use of animals in research.

Sperm collection

Human semen samples were obtained from donors via masturbation after 48 hr of abstinence (Baldi et al., 2022; World Health Organization, 2021). Motile sperm were selected by the swim-up procedure (Baldi et al., 2022) using (noncapacitating) modified human tubal fluid medium with gentamicin–HEPES (mHTF; FUJIFILM Irvine Scientific, Cat#90126) supplemented with 0.5% bovine serum albumin (BSA; Sigma Aldrich, Cat #A7906) at 37°C. Briefly, 1 mL of warm mHTF medium supplemented with 0.5% BSA was placed in a 15-mL sterile conical tube and 0.5–1 mL of semen was gently layered beneath it without mixing. The tube was incubated for 1 hr at a 45° angle at 37°C. After this time, the upper layer containing highly motile sperm was recovered and diluted with 1–2 mL of the same medium. The sample was centrifuged at 400×g for 5 min, the supernatant was discarded and the sperm pellet was resuspended in fresh medium. Sperm concentration was estimated using a Makler chamber and adjusted to 5–10×10⁶ cells/mL.

Mouse sperm were collected from the cauda epididymis of mature CD-1 male mice (12–20 weeks old; Charles River Laboratories, Wilmington, MA). The animals were housed in groups of up to five per cage with free access to food and water and were exposed to a 12-hr light–dark cycle. Animals were sacrificed using a CO₂ chamber followed by cervical dislocation. Cauda epididymides were dissected, small incisions were made

to allow sperm to be released, and the tissue was placed in 500 μ L of noncapacitating medium. Noncapacitating medium consisted of a modified TYH medium (m-TYH), containing: 119.3 mM NaCl, 4.7 mM KCl, 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 1.71 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.56 mM glucose, 0.51 mM sodium pyruvate, 20 mM HEPES, and 10 μ g/mL gentamicin.

Epididymides were incubated for 30 min at 37°C to allow sperm to disperse into the medium. The sperm suspension was then separated from the settled epididymal tissue and transferred to a clean tube. Seven microliters of the sperm suspension was placed on a microscope slide, underneath a 22 $\times$ 22 mm² #1.5 cover glass (VWR VistaVision, 16004-302) and inspected using a phase-contrast microscope at 20 $\times$ magnification to assess concentration and motility and was further diluted with noncapacitating medium to a final concentration of 10 cells/field of view.

Rat sperm were obtained from adult (3- to 6-month-old) male Sprague–Dawley rats. Animals were maintained with food and water ad libitum in a temperature-controlled room with a 12:12 hr light:dark cycle. Spermatozoa were collected from the cauda epididymides. The caudae were dissected, and small incisions were made to release the sperm-rich luminal content from the epididymal duct. The exudate was collected in a conical tube. Rat fertilization medium (RFM, a modification of Toyoda and Chang medium) (Kaplan & Kraicer, 1978) was then gently added over the collected exudate and incubated at 37°C to allow sperm to swim up (Rochwerger & Cuasnicu, 1992).

The RFM consisted of two stock solutions. Solution A contained 87.9 mM NaCl, 5.1 mM KCl, 1.5 mM KH_2PO_4 , 1.5 mM MgSO_4 , 4.28 mM CaCl_2 , 31.25 mM HEPES, and phenol red, adjusted to pH 7.2 with NaOH. Solution B contained 167.1 mM NaHCO_3 and phenol red brought to pH 7.2–7.4 with CO_2 gas. The medium was prepared by mixing 80% solution A and 15% solution B, adding 100 mg/mL glucose, 5.6 mg/mL pyruvate, 4 mg/mL of BSA and penicillin–streptomycin (P0781 Sigma-Aldrich).

After incubation at 37°C, the supernatant containing motile sperm was carefully collected by gentle pipetting, transferred to a clean tube, and further diluted with RFM to a final concentration of 5–10 $\times$ 10⁶ cells/mL.

Bovine sperm were obtained from cryopreserved semen straws of an Aberdeen Angus bull, stored in liquid nitrogen. Straws were then thawed in a water bath at 38°C for 30–45 s. Then, straws were dried with a clean paper tissue, and the ends were cut with sterile scissors carefully expelling the content into a tube containing warm m-TYH medium, supplemented with 0.5% BSA. The tubes containing sperm cells were centrifuged at 300 $\times$ g for 5 min and the supernatant discarded carefully. Motile sperm were selected by the swim-up procedure. For this procedure, after centrifugation, 1–2 mL of warmed medium was gently layered over the sperm cells. Tubes were incubated at 45° angle, at 38°C. After 1 hr, the upper layers containing highly motile sperm were recovered. Then 2–3 mL of the same medium was added, and samples were centrifuged at 300 $\times$ g for 5 min to wash and concentrate the motile sperm fraction, removing residual medium components and ensuring a clean suspension for downstream imaging and tracking (Parrish et al., 1995). The supernatants were discarded, and the sperm pellet was resuspended in fresh medium. Sperm concentration was estimated with a Neubauer chamber and adjusted to 5–10 $\times$ 10⁶ cells/mL.

Induction of human sperm hyperactivation

Hyperactivating conditions were induced by pharmacological elevation of intracellular cAMP using the analog 8-bromoadenosine 3', 5'-cAMP (8-Br-cAMP; 1 mM in mHTF supplemented with 5% BSA at 37°C for 3 hr prior to imaging) which activates signaling pathways known to regulate capacitation-associated changes in sperm motility (Piroozmanesh et al., 2022; Ritagliati et al., 2026; Visconti et al., 2024).

Image acquisition

All samples were imaged at 37°C using a temperature-controlled stage. To ensure reliable segmentation using the pretrained Cellpose models, videos were acquired under phase-contrast illumination with the microscope light intensity adjusted to avoid image saturation, ensuring that sperm heads appeared in gray-scale rather than overexposed white objects, providing sufficient contrast for robust detection. A detailed summary of the microscopy setup and acquisition parameters for each species is provided in Table S1.

Human sperm suspensions (9.8 μ L) were placed on a microscope slide with an 18 $\times$ 18 mm glass coverslip (deltalab), resulting in an approximate chamber depth of 30 μ m. Negative phase-contrast videos were acquired using a Nikon Eclipse E200 microscope with a 10 $\times$ /0.25 Ph1 BM objective and a Basler acA780-75gc camera at 60 frames per second (fps), while positive phase-contrast videos were acquired using a Zeiss AXIO Lab 5 microscope equipped with a 10 $\times$ /0.25 Ph1 objective and a Basler acA1300-200uc camera at 100 fps. Each recording consisted of 240 frames acquired at 60 fps (negative phase contrast) or 400 frames acquired at 100 fps (positive phase contrast), corresponding to 4 s of recording.

Mouse sperm suspensions (30 μ L) were loaded into Leja chamber slides (SC 100-01-02-A CE, 100- μ m depth) and imaged using a Zeiss AXIO Lab A1 microscope. Negative phase-contrast videos were acquired with a Basler acA1920-40um camera and a 20 $\times$ /0.35 Ph1 objective. To achieve 60 fps, the field of view was reduced to 900 $\times$ 800 pixels. Each recording consisted of 400 frames acquired at 60 fps, corresponding to 6.7 s of recording.

Rat sperm suspensions (16.2 μ L) were loaded similarly to human samples, resulting in an approximate chamber depth of 50 μ m, and imaged using a Nikon Eclipse E200 microscope with a negative phase-contrast 10 $\times$ /0.25 Ph1 BM objective and a Basler acA780-75gc camera at 60 fps. Each recording consisted of 240 frames acquired at 60 fps, corresponding to 4 s of recording.

Bovine sperm suspensions (30 μ L) were loaded into Leja chamber slides (SC 100-01-02-A CE, 100- μ m depth) and imaged using the Nikon Eclipse E200 setup described above at 60 fps. Each recording consisted of 240 frames acquired at 60 fps, corresponding to 4 s of recording.

Software

Automated sperm tracking was performed using a Python-based analysis pipeline developed by the authors. The software is openly available on GitHub (<https://github.com/zindrok/automated-sperm-tracking>). An executable graphical user

interface (GUI) is provided together with trained models and representative example datasets. At the time of publication, the automated tracking pipeline supports Cellpose versions 3.1.1 and 4.1.1 for cell detection and Trackpy (Allan et al., n.d.) for tracking. Models trained using a specific Cellpose version must be applied with the same version for subsequent tracking. Together with the software, four trained models are provided for tracking sperm across four different species. All analyses reported in this study were performed using Python version 3.12.5.

The provided executable GUI allows the user to select the input folder containing the videos, define the output folder, and specify the path to the trained Cellpose model. The principal tracking parameters include cell diameter in pixels, maximum linking displacement or search range between consecutive frames, and memory parameter, defined as the maximum number of consecutive frames over which a temporarily undetected sperm cell can remain associated with the same trajectory. The GUI also provides the option to save videos with the reconstructed trajectories overlaid on the original recordings. These videos can be generated for all processed recordings, for a single selected recording, or for a selected subset. Processing can be started, stopped, or reset using the corresponding controls, while a progress bar and processing log provide information on the processing status and the computational device being used.

The output of the GUI is a spreadsheet containing the coordinates generated during tracking. Each row represents a video frame, and each sperm trajectory is represented by a pair of columns labelled x_i and y_i , where i is the trajectory identifier. The values correspond to the horizontal and vertical position of the tracked cell in pixels. In the raw output file, frames in which a sperm cell was not detected or could not be linked to its trajectory are represented by NaN values. A second output file is generated after applying linear interpolation, which fills internal gaps between valid coordinate measurements and produces more continuous trajectories. Interpolation is only applied to gaps bounded by valid detections. Some trajectories may also contain a leading buffer of NaN values before the first valid detection. This buffer can be removed before further analysis. There is no minimum number of frames required for a trajectory to be considered valid in Trackpy. However, for downstream analyses, trajectories may be filtered according to a minimum frame threshold to ensure reliable estimation of motility parameters. MATLAB codes to remove the NaN buffer and filter the trajectories based on a minimum number of frames are provided in the GitHub repository (<https://github.com/zindrok/automated-sperm-tracking>).

Cellpose (Stringer et al., 2021) was used for deep learning-based segmentation of sperm heads. A pretrained model (LC1) was initially applied to individual image frames obtained from sperm motility videos. To adapt the model to the specific imaging conditions and sperm morphologies present in this study, a “human-in-the-loop” training strategy was employed (Supplementary Protocol 1). Briefly, the LC1 model was first applied to experimental image frames, and the resulting segmentation outputs were manually curated by correcting detections and adding missed sperm heads. The curated segmentation masks were then saved and used as training data to retrain the model. This iterative “human-in-the-loop” process, consisting of model prediction, manual correction, and retraining, was

repeated until robust and accurate sperm head detection was achieved.

Due to pronounced differences in sperm head morphology across species, a separate segmentation model was trained for each species. The training strategy was applied independently to human, mouse, rat, and bovine datasets, resulting in species-specific models optimized for their respective morphological and imaging characteristics. The final trained models were used for all subsequent analyses.

Trackpy (Allan et al., n.d.) was used as a Python to link sperm head detections across consecutive frames, enabling reconstruction of individual sperm trajectories. Before tracking, users specify the approximate sperm head diameter, which can be estimated in Fiji (Schindelin et al., 2012). Tracking parameters include the maximum linking distance, which defines the maximum displacement allowed between detections in consecutive frames to be considered the same sperm cell; and the maximum gap-closing frame gap, which defines the maximum number of consecutive frames across which missing detections can be bridged. These parameters should be adjusted according to the acquisition frame rate, image calibration, and the expected sperm displacement between consecutive frames. Tracking parameters were kept constant within experiments for each species but were adjusted between species to account for differences in sperm morphology and motility characteristics, ensuring consistency within datasets while preserving biological relevance.

Trajectory analysis was performed using custom scripts developed by the authors in MATLAB R2023a (MathWorks). One script was used to compute the CASA parameters for each trajectory, and a second script was used to compute the temporally averaged mean square displacement. These scripts are openly available on GitHub at <https://github.com/zindrok/automated-sperm-tracking>.

Results

To enable quantitative analysis of sperm motility across species, we developed an integrated image-analysis pipeline that combines deep learning-based sperm detection, single-cell tracking, and motility quantification within a unified workflow. The pipeline processes time-lapse microscopy videos to identify individual sperm cells, reconstruct their trajectories over time, and extract motility parameters at both the single-cell and population levels. An overview of the complete workflow is shown in Figure 1.

Model training and validation

Species-specific deep learning models were trained to segment sperm heads in human, mouse, rat, and bovine samples. For each species, a dedicated training dataset was generated by manually annotating sperm heads, reflecting the marked interspecies differences in sperm morphology. In total, 5,346 human sperm heads (2,166 from 3 positive phase-contrast videos and 3,180 from 9 negative phase-contrast videos), 2,156 mouse sperm heads (from 83 videos), 1,817 rat sperm heads (from 19 videos), and 3,261 bovine sperm heads (from 19 videos) were manually segmented and used to train the corresponding species-specific models. After training, each model was applied to held-out image

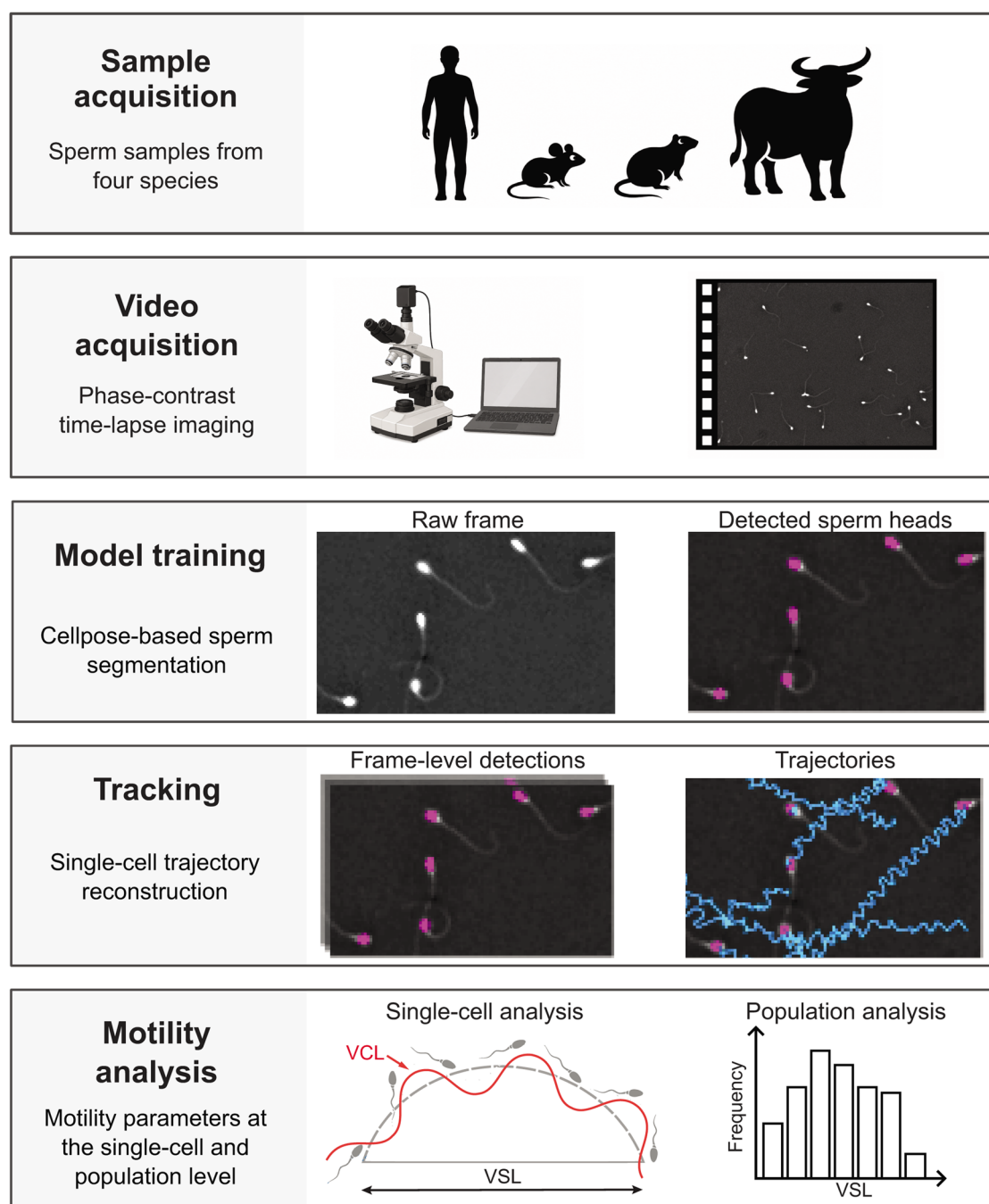


Figure 1 Overview of the sperm tracking and motility analysis system. Sperm samples from multiple species were imaged using time-lapse microscopy and processed through a workflow that includes training, detection, tracking, and motility parameter extraction. *Note.* VSL = straight-line velocity.

datasets to assess segmentation performance. Representative examples of sperm head detections for each species are shown in Figure 2, illustrating accurate segmentation across species regardless of head size and shape. The agreement between the segmentation mask and the underlying sperm head morphology can be further appreciated in the figure insets where a zoom of the image is shown for each representative field of view.

To quantitatively validate model performance, standard detection metrics were computed based on comparisons between automated detections and manually annotated ground truths. Validation was performed on independent

held-out datasets, not used for model training, comprising images from 8 videos for human negative phase contrast (307 sperm cells), 6 videos for human positive phase contrast (182 sperm cells), 35 videos for mouse (263 sperm cells), 16 videos for rat (555 sperm cells), and 12 videos for bovine (591 sperm cells). Correctly detected sperm heads were classified as true positives (TP), detections corresponding to nonsperm objects were classified as false positives (FP), and sperm heads that were not detected by the model were classified as false negatives (FN). From these values, recall, precision, and F1 score were calculated.

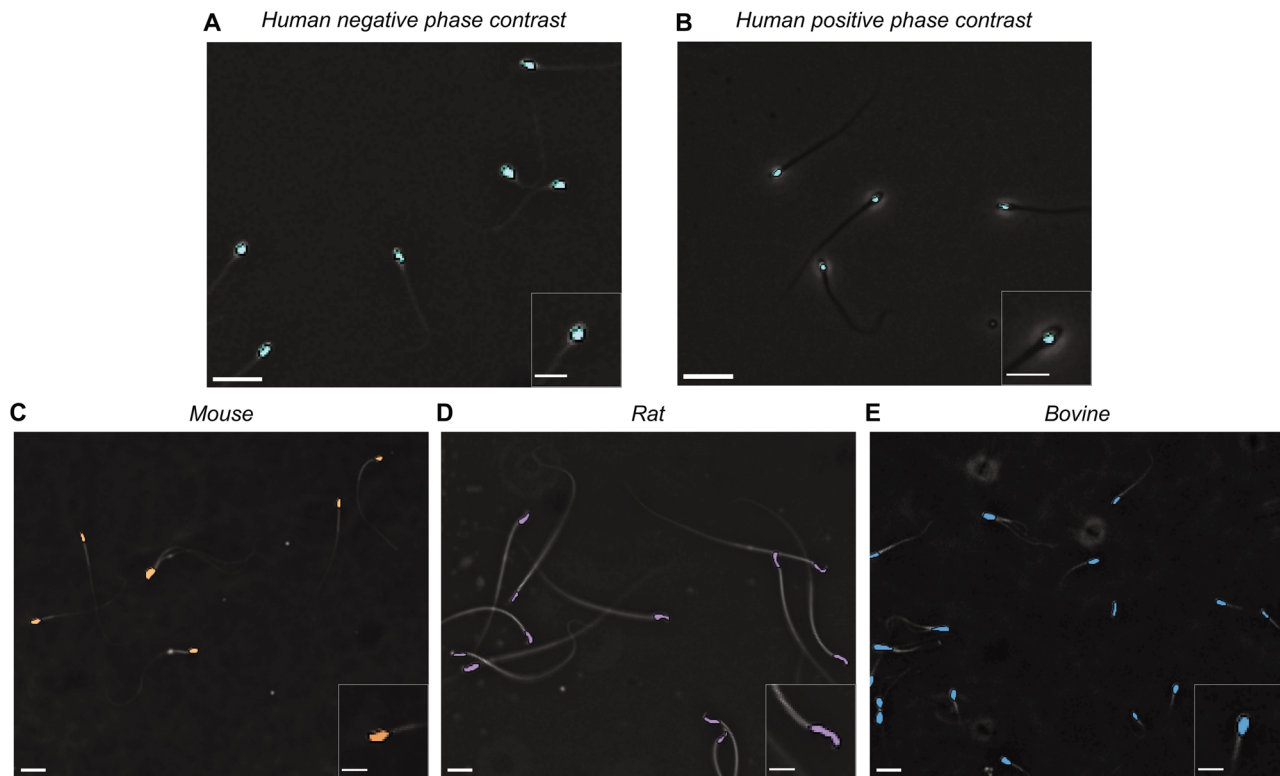


Figure 2 Species-specific sperm head detections. Representative single frames from sperm videos are shown for each species, with the corresponding detection masks overlaid: (A) human sperm imaged with negative phase contrast; and (B) human sperm imaged with positive phase contrast. Images were obtained with negative phase contrast for (C) mouse sperm; (D) rat sperm; (E) bovine sperm. The overlaid masks show qualitative good agreement with the sperm head morphology. Insets display a magnified view of an individual cell to highlight the detected structure, corresponding to the sperm head. Scale bars: 20 μm in main panels; 10 μm in insets.

Recall was defined as the fraction of sperm heads that were correctly detected, reflecting the sensitivity of the model. Precision was defined as the fraction of detections that corresponded to correctly classified sperm heads, reflecting specificity. The F1 score, calculated as the harmonic mean of recall and precision, provided an overall measure of detection performance. Namely,

$$\text{Recall} = \frac{TP}{TP + FN}, \quad (1)$$

$$\text{Precision} = \frac{TP}{TP + FP}, \quad (2)$$

$$\text{F1 score} = \frac{2(\text{Precision} \times \text{Recall})}{\text{Precision} + \text{Recall}}. \quad (3)$$

Quantitative validation results for each species are summarized in [Table 1](#) for each species. Across all species, the models achieved high recall and precision values, indicating robust and reliable sperm head detection and supporting their use as input for downstream trajectory reconstruction. Notably, rat sperm represent a particularly challenging case for CASA systems due to their elongated head morphology and frequent clustering, which often compromise detection accuracy. Despite these inherent difficulties, our model maintains high performance in rat samples, highlighting its robustness and establishing a reliable tool for sperm analysis across species.

Sperm tracking

Automated sperm tracking was performed by integrating the deep learning-based sperm head detections with a tracking framework. Specifically, sperm head segmentations were used as input for trajectory reconstruction using Trackpy, employing the Linear Assignment Problem (LAP) tracker ([Jaqaman et al., 2008](#)). This approach enabled robust reconstruction of individual sperm trajectories under heterogeneous motility conditions. The pipeline was successfully applied to sperm across the four species described above. Representative examples of reconstructed sperm trajectories for each species are shown in [Figure 3](#), illustrating the ability of the pipeline to track sperm cells with distinct motility patterns and morphological features across species. Videos corresponding to the trajectories in these images are presented in the [Supplementary material](#).

Human sperm motility was evaluated under basal and hyperactivation-induced condition ([Brisard et al., 2025](#); [Visconti et al., 2024](#); [Yanagimachi, 1994](#)). A total of 338 trajectories from 7 videos of sperm in basal conditions and 505 trajectories from 11 videos from hyperactivation-condition were analyzed. Representative sperm trajectory examples under both conditions are shown in [Figure 4](#), where hyperactivation is characterized by more erratic, high amplitude, and less linear movement patterns, in response to this incubation condition. In addition, 179 trajectories from 14 mouse videos, 2,186 trajectories from 77 rat videos and 998 trajectories from 29 bovine videos were reconstructed to

further validate the robustness of the tracking approach across datasets.

Motility analysis

For all analyses presented here, trajectories were standardized to a length of 64 frames at 60 fps to facilitate direct comparisons among datasets and to provide a uniform temporal window for motility estimation. The trajectory dataset used in these analyses has been made publicly available in the GitHub repository. To quantitatively characterize sperm motility from the obtained trajectories, we calculated selected standard CASA kinematic parameters and metrics commonly used in random-walk analysis.

Table 1 Detection performance of the Cellpose-based segmentation model across species.

Species	Recall	Precision	F1 score
Human (negative contrast)	0.98	0.98	0.98
Human (positive contrast)	0.94	0.99	0.97
Mouse	0.94	0.97	0.96
Rat	0.92	0.99	0.96
Bovine	0.97	0.96	0.97

Note. Recall, precision, and F1 score are reported for human, mouse, rat, and bovine sperm using independent validation datasets.

Despite its limited scope, this analysis provided a proof of concept for the applicability and versatility of our framework. Given a trajectory with positions r_i recorded at times t_i with $i=0, 1, \dots, N$, we considered three measures typically reported by CASA systems: the “straight-line velocity” (VSL), the “curvilinear velocity” (VCL), and the “amplitude of lateral head displacement” (ALH). The VSL is defined as the end-to-end average velocity,

$$VSL = \frac{|r_N - r_0|}{t_N}, \quad (4)$$

where r_0 is initial position and t_N and r_N are the final time and position, respectively. The VCL is average instantaneous velocity,

$$VCL = \frac{\sum |r_{i+1} - r_i|}{t_N}. \quad (5)$$

ALH quantifies the side-to-side movement of a sperm head, reflecting flagellar beat intensity.

Figure 5 shows the histograms of these three parameters typically reported by CASA systems for the four analyzed species. All samples were analyzed under noncapacitating conditions. Human sperm were also treated with 8-Br-cAMP to induce hyperactivation. The histograms illustrate the strong heterogeneity that can exist within a sample. Moreover, for human sperm, clear differences are observed between the control and hyperactivated conditions. In particular, treatment with 8-Br-cAMP resulted in a shift toward higher values of VCL and ALH_{max} , consistent with the increased flagellar amplitude and vigor characteristic of hyperactivated motility.

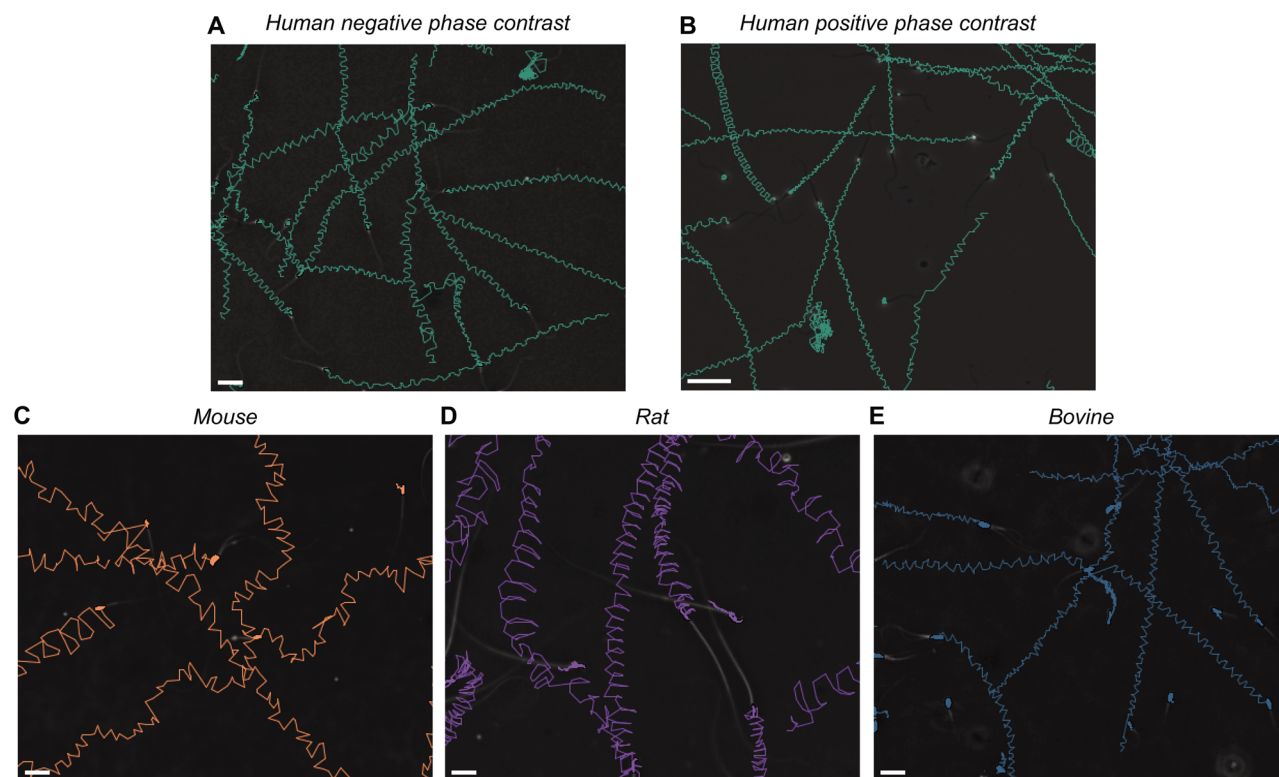


Figure 3 Sperm trajectories. Representative tracking outputs generated using the proposed analysis pipeline for human, mouse, rat, and bovine sperm. Colored lines indicate sperm trajectories reconstructed from time-lapse microscopy data. (A) human sperm imaged with negative phase contrast; (B) human sperm imaged with positive phase contrast; (C) mouse sperm; (D) rat sperm; (E) bovine sperm. Scale bars: 20 μ m.

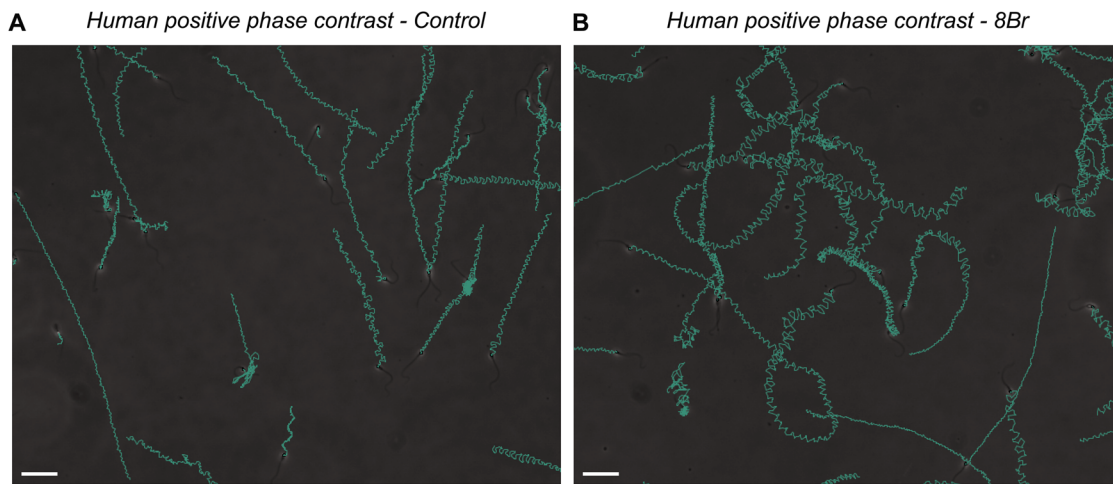


Figure 4 Representative human sperm trajectories under basal (A) and hyperactivated conditions (B). Hyperactivated sperm exhibit more erratic, high-amplitude, and less linear trajectories compared with control cells, consistent with characteristic changes in flagellar beating and motility patterns. Scale bar 20 μm . Note. 8Br = 8-bromoadenosine 3', 5'-cAMP.

Beyond these three standard CASA parameters, we extended our quantitative analysis to a commonly used metric to characterize particle dynamics, namely the mean square displacement [MSD (Manzo & Garcia-Parajo, 2015; Weigel et al., 2011; Zaferani et al., 2021; Zaferani & Abbaspourrad, 2023)]. The MSD of an individual trajectory is defined as

$$\overline{\delta^2(m\Delta t)} = \frac{1}{N-m+1} \sum_{i=0}^{N-m} |r(t_{i+m}) - r(t_i)|^2, \quad (6)$$

for a lag time $t = m\Delta t$, where the overbar denotes a temporal average, $\delta(t)$ is displacement in time t , Δt is the time between frames and m is a positive integer. The MSD quantifies the average squared displacement of sperm over increasing time intervals, providing insight into their motility behavior across timescales (Pacheco-Pozo et al., 2026).

Figure 6 shows the MSD of 25 randomly selected trajectories together with the MSD averaged over all the trajectories as a function of time. Two distinct modes of motion can be identified. At short timescales the MSD reflect local oscillatory motion associated with flagellar beating rather than net displacement. At longer timescales, the MSD increases at a different rate, corresponding to effective displacement and sustained sperm movement. In human sperm, treatment with 8-Br-cAMP results in higher MSD values across timescales, consistent with enhanced motility under hyperactivating conditions.

Benchmarking against CASA for human sperm analysis

To validate the proposed method, we compared the motility parameters derived from our tracking pipeline with those obtained using the Sperm Class Analyzer (SCA v.6.2.0.1.; Microptic SL), a commercial CASA system, on the same set of human sperm videos. The videos were obtained using the SCA system and consisted of 30 frames captured at 60 fps. For the benchmark comparison, our tracking pipeline was applied to the same 30-frame videos generated and analyzed by the SCA system. The resulting trajectories were then analyzed using the motility-analysis procedure described above, restricted to trajectories of exactly 30

frames each. This ensured that both methods were evaluated on the same video data. Computer-assisted sperm analysis remains a widely used reference standard in both clinical and research settings for the assessment of sperm kinematics. Equivalent motility descriptors were extracted from both approaches, including VSL, VCL, and ALH. The motility descriptors from the SCA system were extracted from the report sheet, whereas the corresponding descriptors from our method were computed from the trajectories generated by our tracking pipeline. The SCA system reports the maximum of the ALH along the trajectory; therefore, we use that value for comparison. As the SCA system excludes immobile trajectories, the analysis was restricted to trajectories with $\text{VSL} > 10 \mu\text{m/s}$. The mean values obtained with the CASA system and our tracking are compared in Table 2 to assess agreement and validate the motility parameters derived from our trajectory analysis. The standard deviations of the obtained parameters are also reported in Table 2. The results show that VSL, VCL, and ALH obtained with our method and with CASA fall within the same range, suggesting comparable results for these descriptors. A formal analysis to evaluate whether the two measurement methods produce statistically similar results was not performed because the commercial CASA system employed provides only mean values and does not allow access to the corresponding individual trajectories or dispersion measures for its reported values.

Discussion

The present study describes the development of an open-source framework for sperm detection, tracking, and motility analysis. By combining deep learning-based detection with automated tracking, this approach enables the reconstruction of individual sperm trajectories from any microscopy recordings. Rather than relying on summary metrics alone, this framework allows direct access to individual trajectories. Importantly, the shift from population-averaged descriptors to trajectory-resolved analysis is not only methodological but also conceptual, as it reveals features of sperm behavior that remain hidden in conventional analyses. Benchmarking against a commercial CASA system demonstrates the consistency of our method for standard motility

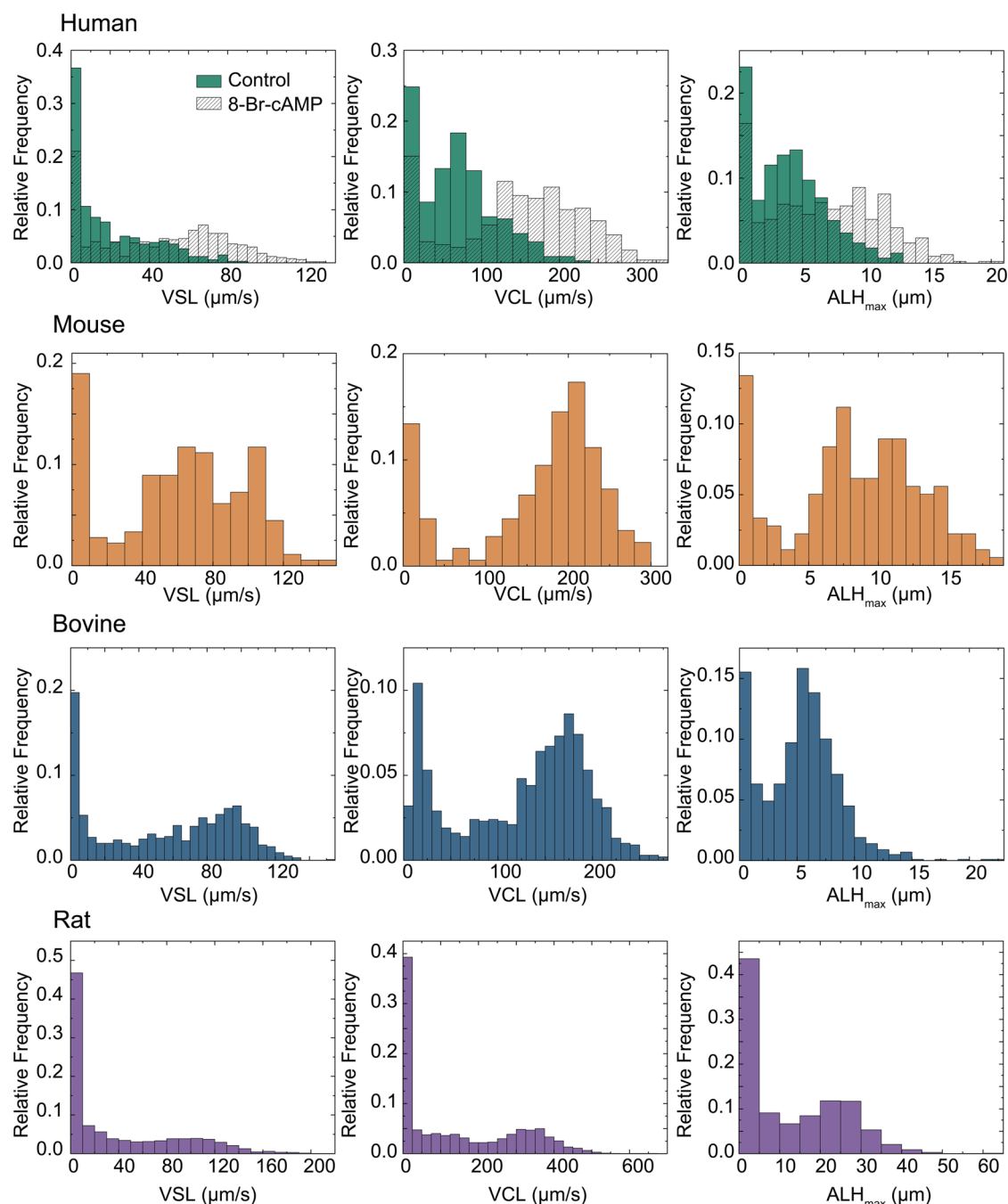


Figure 5 Computer-assisted sperm analysis (CASA)-like motility parameters. Histograms showing the distributions of three CASA-like motility parameters—straight-line velocity (VSL), curvilinear velocity (VCL), and maximum amplitude of lateral head displacement (ALH)—across four species: human, mouse, bovine, and rat. Human samples included noncapacitated (basal) sperm and sperm treated with 8-bromoadenosine 3', 5'-cAMP (8-Br-cAMP) to induce hyperactivation. For mouse, bovine and rat, only noncapacitated samples were analyzed.

descriptors while underscoring the expanded analytical potential of our framework. Direct access to individual sperm trajectories enables the computation of a wider range of motility metrics and provides a flexible foundation for developing and evaluating novel quantitative measures of sperm movement.

A key strength of this platform is its adaptability. Because sperm detection relies on a trainable model, the system can be adapted to different imaging setups and cell types with relatively limited training data. This is particularly relevant for

sperm imaging, where variations in cell density, debris, and contrast often make standardized analysis challenging. In the present study, the framework was applied to four different species, using for each case the phase-contrast modality that best matched the characteristics of the dataset. This highlights the flexibility of the approach and supports its potential extension to additional species and across multiple experimental systems. Moreover, acquisition parameters such as video duration and frame rate, as well as trajectory filtering criteria, can be adjusted

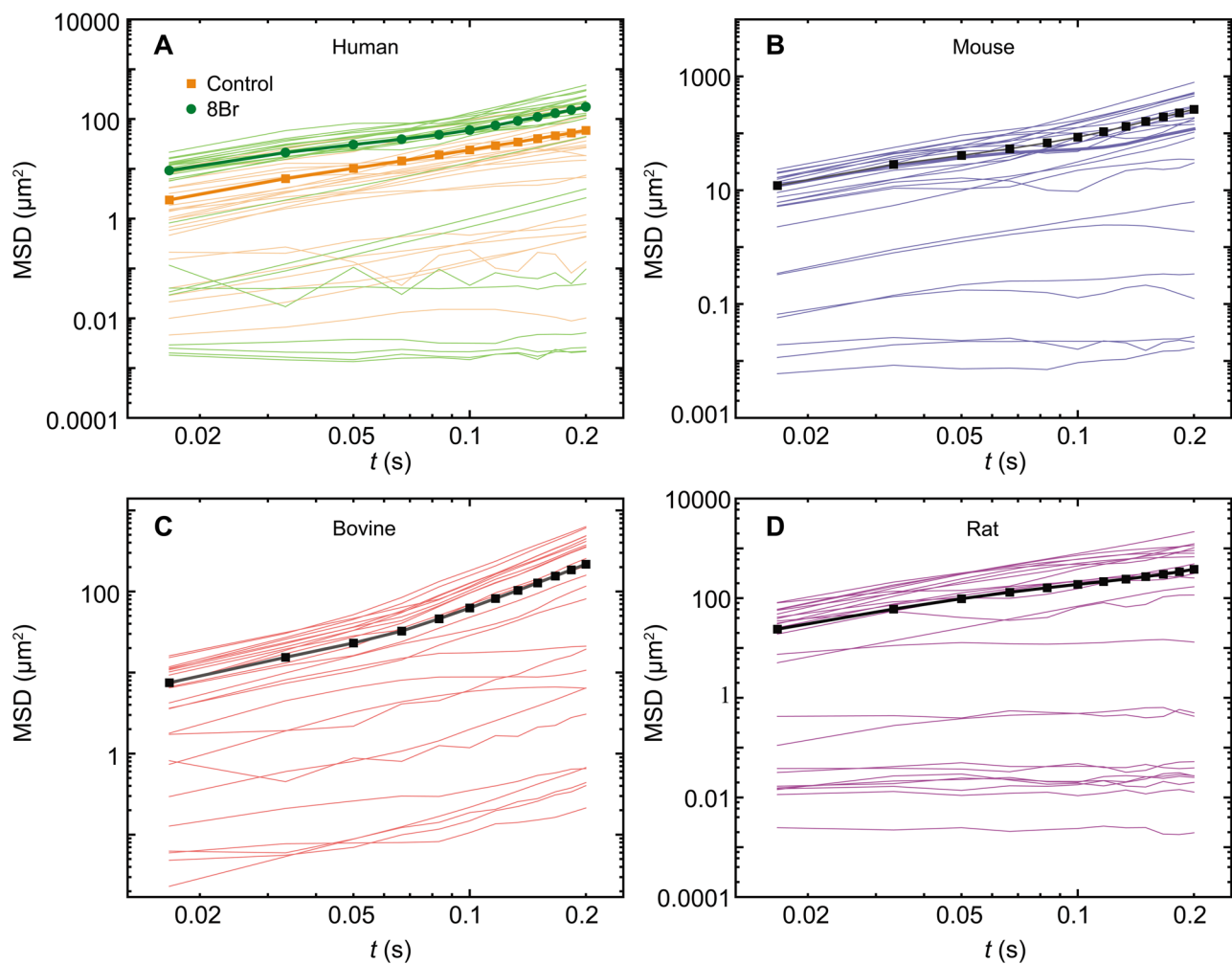


Figure 6 Mean squared displacement (MSD) vs time for human, mouse, bovine, and rat sperm. Thin lines show MSD curves of 25 randomly selected individual trajectories for each condition, while symbols and thick lines show the MSD averaged over all available trajectories. (A) Human samples included noncapacitated (control) sperm and sperm treated with 8-bromoadenosine 3', 5'-cAMP (8Br) to induce hyperactivation. For (B) mouse, (C) bovine, and (D) rat, only samples in noncapacitating conditions were analyzed.

Table 2 Comparison of motility parameters obtained with our tracking-based method and conventional CASA for the same set of human sperm videos under basal conditions.

Parameters	Our method	CASA
VSL (μm/s)	43.85 ± 21.84	40.28
VCL (μm/s)	87.35 ± 30.26	93.02
ALH _{max} (μm)	1.88 ± 1.15	1.94

Note. Mean ± standard deviation is reported for our method, whereas the CASA system provides average values only. CASA = computer-assisted sperm analysis; VSL = straight-line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement.

depending on the biological question, enabling for example, the selection of long trajectories or the isolation of specific motility subpopulations.

Another advantage of the proposed framework is the accessibility of the output data. In contrast to many commercial systems, which typically provide restricted or highly processed outputs, this workflow generates trajectory-level datasets that can be directly explored and analyzed using widely adopted

computational environments such as Python or R. This flexibility makes it easier to integrate sperm motility data with downstream approaches such as statistical modeling or machine learning, and to test alternative ways of describing motility. Importantly, the proposed method may facilitate the identification of novel quantitative descriptors of sperm function based on motility patterns, potentially improving how sperm functional competence is assessed.

Working at the level of individual trajectories reveals aspects of sperm behavior that are often lost in conventional analysis. This trajectory-resolved analysis enables the characterization of individual movement patterns and their variability across cells and time, potentially providing a richer description of sperm motility behavior. In our data, this variability is reflected in the emergence of multimodal distributions of motility parameters under hyperactivating conditions (Figure 5), supporting the coexistence of distinct subpopulations rather than a uniform behavioral shift (Buffone et al., 2004; Martínez-Pastor, 2022; Valverde et al., 2019). Capturing this variability is important, particularly in the context of capacitation, where both motility and underlying signaling states evolve over time and not all cells respond synchronously (Cohen-Dayag et al.,

1995; Luque et al., 2018). These observations are consistent with recent advances in sperm analysis that emphasize the importance of trajectory-level information. Previous work has shown that the extraction of kinematic parameters, such as amplitude of lateral head displacement and beat cross frequency, is highly sensitive to tracking accuracy (Shahali et al., 2026a). While such approaches improve parameter estimation and enable trajectory-based classification, our results further suggest that analyzing the full distribution of motility behaviors provides additional insight into the heterogeneity of sperm responses. Similarly, deep learning-based frameworks have been successfully applied to classify sperm motility patterns directly from trajectory data, achieving high accuracy in distinguishing behavioral states (Shahali et al., 2026b). In this context, our approach complements these strategies by enabling detailed exploration of trajectory variability without restricting the analysis to predefined classes. More broadly, our findings align with emerging single-cell approaches that combine tracking and machine learning to extract increasingly complex features of sperm behavior (Khanal et al., 2021; Simons & Olson, 2018). Recent studies have demonstrated the potential of convolutional neural networks to capture additional dimensions of motility, including three-dimensional motion and rolling behavior, highlighting the richness of information accessible through high-resolution tracking (De Clemente et al., 2025). Together, these approaches point toward a shift from population-averaged descriptors to more comprehensive, single-cell-resolved analyses of sperm function (Hart et al., 2026; Horenin et al., 2024; Martínez-Pastor et al., 2011; Ramón & Martínez-Pastor, 2018).

Despite the advantages discussed, some limitations should be considered. The acquisition of videos and trajectory reconstructions can generate large datasets, which can quickly become demanding in terms of storage and processing time. Although deep learning models require an initial investment in training and parameter optimization, this step has already been completed for the four species included in this study, and the corresponding pretrained models will be made available to users. Therefore, routine use of the framework does not require model retraining. Training is only necessary when adapting the framework to datasets that differ substantially from the provided models, such as images from additional species or acquired under markedly different imaging conditions. While these steps are not prohibitive, they may represent a challenge for laboratories seeking to develop custom models without prior experience in computational image analysis.

From clinical and technological perspectives, our results also support the growing recognition that traditional CASA systems present important limitations. In addition to issues related to cost and accessibility, CASA-based measurements may introduce systematic inaccuracies and variability, particularly under diverse experimental conditions (Villani et al., 2022). In addition, the way this information is typically reported may not be informative in research settings, given that values are usually averaged and fluctuations are often not provided. As a result, most studies with CASA systems rely primarily on the percentage of motile or progressively motile sperm, a practice that has remained largely unchanged for many years. Recent artificial intelligence-based approaches have demonstrated improved precision, repeatability, and agreement with manual tracking, outperforming conventional CASA systems in the estimation of motility parameters (Krapf et al., 2016; Muñoz-Gil et al., 2025; Weron et al., 2017).

Within this landscape, open and customizable frameworks such as the one presented here may provide a valuable alternative for both research and, potentially, clinical applications.

Future work may further extend the applicability of this approach. The open architecture of the platform allows its integration with additional analytical tools, including classification models, biophysical modeling, and advanced statistical frameworks (Krapf et al., 2016; Muñoz-Gil et al., 2025; Weron et al., 2017). An important next step will be to link motility patterns with functional readouts, such as capacitation state, to better understand how dynamic sperm behavior relates to fertilization potential. Moreover, the adaptability of the detection and tracking modules may facilitate application to other cell types or experimental systems. Together, this framework provides a flexible and transparent platform to investigate sperm motility at single-cell resolution, with the potential to advance both fundamental understanding and the development of improved diagnostic and predictive tools in male infertility.

Supplementary material

Supplementary material is available at *Reproduction* online.

Author contributions

Di.K., M.G.B., and Da.K. designed and supervised the study, P.A., E.K., A.M.K., A.M.V., A.Romarowski, and A.R.C. performed experiments; A.Rajesh, H.O.H., and A.P.-P. wrote computer codes; E.K., C.C., A.M.K., and A.P.-P. analyzed the data and performed statistical analysis; A.R.C. and P.S.C. contributed biological materials; Di.K., M.G.B., P.A., E.K., A.M.V., A.Romarowski, and A.P.-P. contributed to the interpretation of the results. P.A., E.K., A.P.-P., and Di.K., prepared the manuscript with contributions of all other authors.

Conflicts of interest

The authors declare no conflicts of interest.

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Data availability

Data supporting the results of this study can be obtained from the corresponding author.

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