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An interdisciplinary systems approach to study sperm physiology and evolution

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Optical trapping is a noninvasive biophotonic tool that has been developed to study the physiological and biomechanical properties of cells. The custom-designed optical system is built to direct near-infrared laser light into an inverted microscope to create a single-point three-dimensional gradient laser trap at the microscope focal point. A real-time automated tracking and trapping system (RATTS) is described that provides a remote user-friendly robotic interface. The combination of laser tweezers, fluorescent imaging, and RATTS can measure sperm swimming speed and swimming force simultaneously with mitochondrial membrane potential (MMP). The roles of two sources of adenosine triphosphate in sperm motility/energetics are studied: oxidative phosphorylation, which occurs in the mitochondria located in the sperm midpiece, and glycolysis, which occurs along the length of the sperm tail (flagellum). The effects of glucose, oxidative phosphorylation inhibitors, and glycolytic inhibitors on human sperm motility are studied. This combination of photonic physical and engineering tools has been used to examine the evolutionary effect of sperm competition in primates. The results demonstrate a correlation between mating type and sperm motility: sperm from polygamous (multi-partner) primate species swim faster and with greater force than sperm from polygynous (single partner) primate species. In summary, engineering and biological systems are combined to provide a powerful interdisciplinary approach to study the complex biological systems that drive the sperm toward the egg. © 2010 John Wiley & Sons, Inc. *WIREs Syst Biol Med* 2011 3 36–47 DOI: 10.1002/wsbm.106

INTRODUCTION

A swimming sperm can be viewed as a complex 'organism' that in order to achieve its ultimate purpose (fertilization of an egg) must successfully integrate an array of complex biochemical and molecular pathways that control energy generation, movement of the flagellum, and the final interaction with the egg. The pathways associated with all these must function in an integrated fashion to achieve the ultimate result. How we study these systems, whether as individual subsets of molecular signals and 'organism' behaviors or as an integrated pattern of signal transduction events, will determine how far we advance our understanding of these complex systems. New engineering approaches, such as optical manipulation, computer-based robotic control of systems, and image-based analysis and computation, can be combined to provide a powerful interdisciplinary approach to study the complex biological systems that drive the sperm toward the egg.

Optical Trapping (Laser Tweezers) and Sperm Motility

Optical trapping (tweezers) was first described by Arthur Ashkin in 1970[1] and was first applied to the manipulation of whole cells in 1987.[2] Subsequently,

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laser tweezers were successfully used to move individual organelles inside the cell, such as chromosomes[3] and plant cytoplasmic filaments.[4] A beam of light is composed of photons, each of which carries a momentum that is a function of wavelength. When a photon is incident upon a particle, there is a transfer of momentum through scattering. If the laser light is focused to a small area by a microscope objective, for example, the scattering photons will exert a radiation pressure that will result in an optical force. These forces can be significant if the particle is small, and can either push or pull (trap) the particle depending upon the optical geometry of the trap and the object being trapped.[5]

It was not long after, that individual sperm were optically trapped and released as a method to determine their relative swimming force[6] and as a means to study swimming abnormalities as they relate to problems of infertility.[7–10] These studies determined that the minimum amount of laser power needed to hold the sperm in the trap (or the threshold escape power) is directly proportional to the sperm's swimming force $F = Q \times P/c$, where F is the swimming force, P is the laser power, c is the speed of light in the medium, and Q is the geometrically determined trapping efficiency parameter.[9] In addition, studies on the relationship between sperm velocity and swimming force (escape power from trap) were described for human and dog.[11–12]

Compared to existing devices, primarily computer-assisted sperm analysis (CASA) systems for measuring sperm motility (primarily swimming velocity), the laser trap presented the opportunity to not only examine the single sperm motility in detail, but the possibility of measuring individual sperm swimming force, the energetics of the individual sperm, and the ability to determine if there are correlations between swimming velocity, force, and fertilization potential. Applications would apply not only to gaining a better understanding of sperm physiology and motility, but also could result in more comprehensive assays for abnormal sperm behavior in the case of infertility, development of better methods for cryopreservation and storage of sperm, and the development of potential drugs/therapies for improving the motility and ultimately the fertilizing capacity of sperm from low fertility males. In addition, the technology can be applied to improving/understanding fertility in endangered species and animal husbandry.

TRACKING AND TRAPPING A FAST MOVING OBJECT (SPERM)

In the early studies, the experimenter would have to constantly look at the sperm swimming patterns on the images captured by the CCD camera and manually move the joystick that connects to the x–y microscope stage in order to relocate the sperm-of-interest in the laser trap, then depress the top button on the joystick which connects to the mechanical shutter to turn on the laser trap. This was relatively easy for slow-swimming sperm and virtually impossible for faster (100+ μm/second) sperm. In addition, in many experiments it was necessary to record the swimming velocity both before and after the sperm was trapped, and as will be seen in a later section, monitor the cellular respiration before, during, and after laser trapping. Therefore, new computer software and robotic interfaces with hardware had to be developed.

Most sperm trapping experiments involve single sperm studies and require manually driven video analysis in a frame-by-frame manner in order to measure swimming velocities before and after trapping. Additionally, stable three-dimensional (3D) laser trapping requires a high numerical aperture (NA) oil-immersion objective lens, which characteristically has a very shallow depth of field (a few micrometers). As a result, swimming sperm routinely move in and out of focus as compared to imaging with a low NA air-immersion objective lens. Therefore, it became necessary to develop a more robust automated sperm tracking algorithm for single sperm trapping studies.

The solution to this problem was the development of a real-time automated tracking and trapping system (RATTS) that operates at video rate and provides remote robotic interfaces with the hardware (Figure 1(a) shows the block diagram of the layout[13]). The optical system and specimen preparation have been detailed previously.[12] Briefly, cryogenically frozen dog sperm were thawed and suspended in Biggers, Whittens, and Whittingham (BWW) media and injected into 2-mm deep cell culture chambers. An Nd:YVO4 continuous wave 1064-nm wavelength laser is focused into a Zeiss Axiovert S100 microscope equipped with a phase III, NA 1.3 oil-immersion objective. The laser creates a single-point 3D gradient laser trap. An image acquisition board is housed in the host computer to digitize analog video signals from the camera. A motion controller is used to control the x–y stepper motor stage of the microscope. A mechanical shutter in the laser path is controlled by a shutter driver through two lines of digital input–output from the motion controller. A rotary stepper motor mount housing a Glan laser linear polarizer is controlled by the motion controller to modulate power in the laser trap.

A key feature of RATTS is the ability to track sperm at video rates and update the microscope stage position to keep a swimming sperm in the field
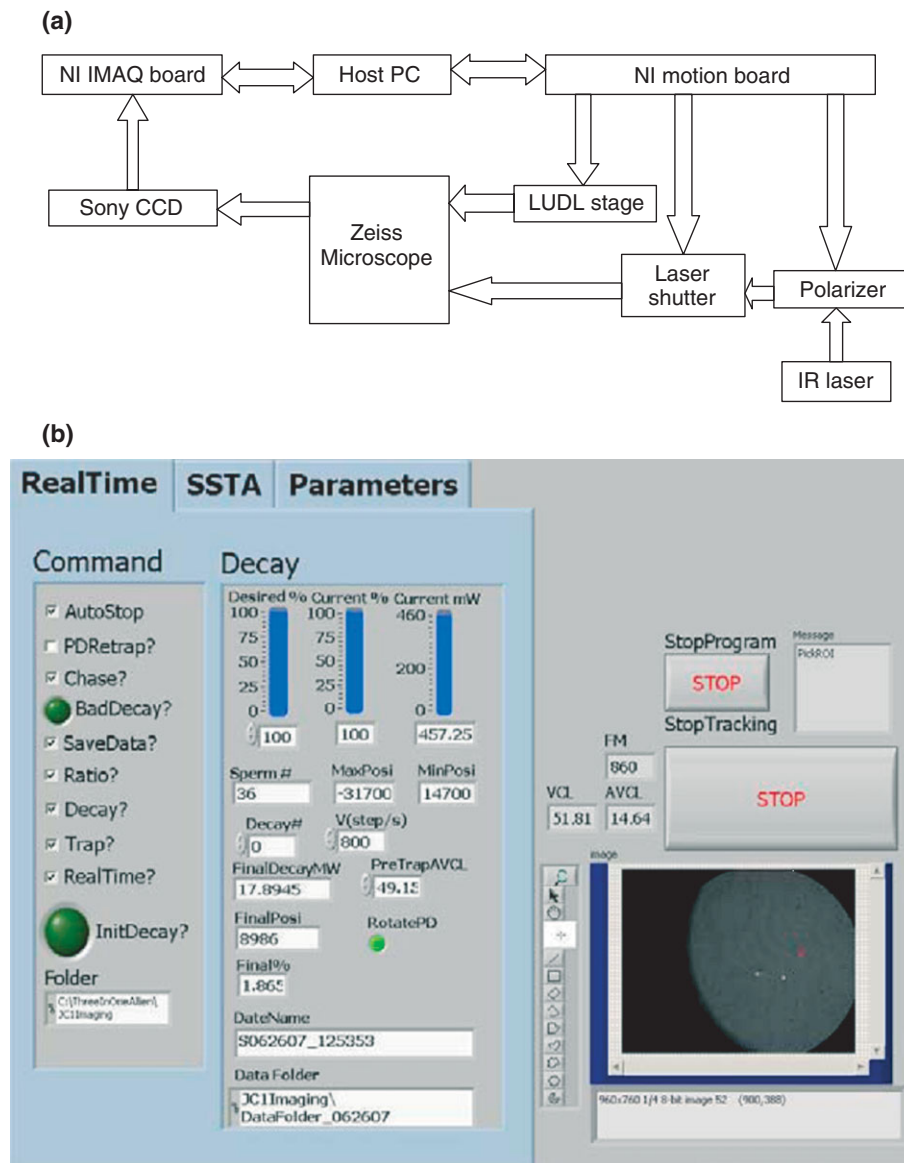
(a)

(b)

FIGURE 1 | (a) Hardware design of the real-time automated tracking and trapping system (RATTS) system. (b) Front Panel of RATTS: during the trapping phase of the experiment, RATTS implements an escape detection subroutine to detect the presence of a sperm in the laser trap and to respond if the sperm escapes the trap. In power decay experiments, the laser power used to first trap a sperm is a user-defined percentage of maximum power. (Reprinted with permission from Ref 17. Copyright 2008 Springer Science+Business Media).

of view. RATTS is custom coded in the LabView language to process streaming images, calculate sperm trajectories, and drive the motion hardware. RATTS implements the image segmentation and single sperm tracking algorithm (SSTA) previously described.[14] As an improvement over commercial CASA systems, SSTA recognizes sperm collisions and uses its post-collision analysis to statistically recover the tracked sperm. A post-collision analysis is trained with non-colliding sperm trajectories to statistically determine the probability of deviating from mean swimming behaviors. These probabilities determine

the likelihood of sperm pairing between pre- and post-collision sperm.

The automated trapping feature of RATTS replaces the earlier manual protocol.[12] User input is limited to setting parameters prior to an experiment and selecting, via the computer mouse, a sperm in the field for analysis. The user can preselect the method of laser exposure: (1) laser power is held constant for a fixed duration in the trapping phase of the experiment or (2) laser power is decayed during trapping. During the experiment, the user selects a sperm to be analyzed by clicking on its image with the arrow cursor on the

front panel of RATTS as shown in Figure 1(b). The cursor coordinate is registered, passed to the tracking algorithm, and computation proceeds with no further intervention. Once the specified number of frames has been processed, the stage is moved to place the sperm under the laser trap and the shutter is opened.

Using RATTS, sperm are tracked for extended durations before and after laser trap experiments. Motility measurements including VCL and the absolute position of the sperm relative to the cell chamber are calculated and written to the hard drive at video rates. Experimental throughput is increased over 30 times compared to off-line data analysis.[12] Figure 2(a) and (b) plots the trajectories of two different sperm which have been tracked for as many as 11,167 consecutive video frames. Figure 2(c) and (d) demonstrates the real-time sperm tracking with trapping at constant laser power. Sperm were trapped for 200 video frames (6.67 seconds) at 350 mW laser power and trajectories after the laser is turned off are also recorded.[13]

COMBINING TRACKING, TRAPPING, AND FLUORESCENT RATIO MEASUREMENTS TO STUDY ENERGETICS IN A HIGHLY MOTILE CELL SYSTEM (SPERM)

Ratiometric Fluorescent Probe for Sperm Physiology Study

The idea of monitoring sperm physiology (respiration) before, during, and after laser trapping would be extremely useful, yet a challenge to accomplish. The approach that was developed used a two-wavelength ratiometric fluorescent probe that measured changes in mitochondrial membrane potential (MMP) located in a sperm's midpiece, the presumed energy center of the sperm—where the bulk of adenosine triphosphate (ATP) is generated via aerobic respiration. The particular dye used, DiOC2(3) (3,3′-diethyloxacarbocyanine iodide), monitors the voltage potential across the inner membrane of the mitochondria. It is a cationic cyanine dye that primarily
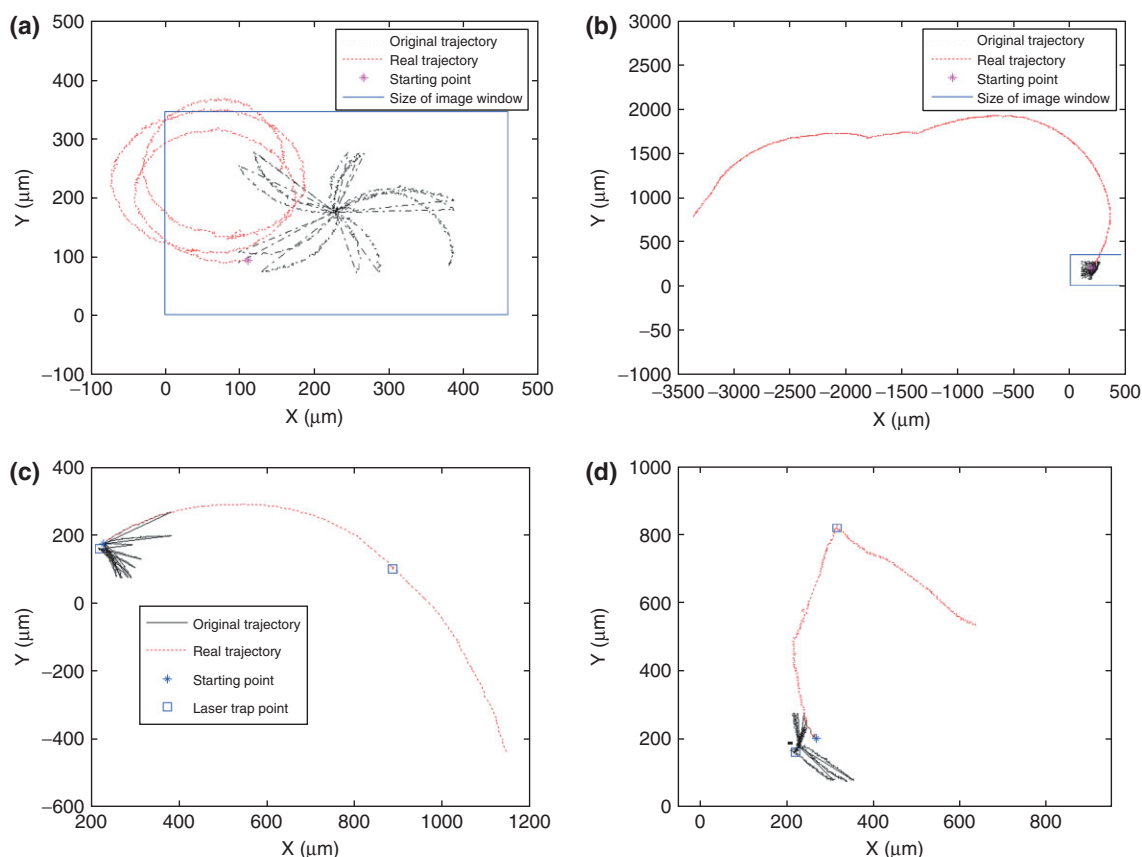

FIGURE 2 | Trajectories of real-time continuously tracked (a, b) and track-trapped (c, d) sperm.[13] Continuous tracks are blotted for both absolute (dashed line) and 'real' coordinates, and for field-of-view (solid line) and 'original' coordinates. Straight-line black segments represent stage movements. For each trajectory, the number of consecutive frames (Frame#) and the average curvilinear velocity (VCL) are shown in (a) and (b). In the track-trapped case, for each trajectory the starting point (asterisks) and the trapping location (squares) are shown in (c) and (d).

accumulates in the mitochondria of a cell in response to the electrochemical proton gradient. The probe emits both a red (high proton gradient) and green fluorescence (low proton gradient). The ratiometric parameter (red/green intensity) is a size-independent measure of MMP, as the green fluorescence varies with size and red fluorescence is dependent on both size and MMP.

Optical Setup

The optical microscope system and laser trap are similar to that described earlier in this article as shown in Figure 3. The imaging setup was adapted from previous studies.[15] Two dual-view video adapters are used to incorporate the laser into the microscope and simultaneously image the sperm in phase contrast and fluorescence. For the fluorescent images, a dual-view system splits the red and green fluorescent light emitted by the specimen to produce a copy of the image for each color. Fluorescent emission filters are placed in this emission-splitting system (green fluorescence emitter: HQ 535/40-nm M filter; red fluorescence emitter: HQ 605/50-nm M filter). The dual-view system is coupled to a digital camera (Quantix 57) that captures the fluorescent images.[16]

Hardware System and Software Algorithm

RATTS has been modified to measure MMP (before, during, and after laser trapping) in conjunction with swimming speed and escape laser power of individual sperm.[17] Figure 4 shows the system hardware diagram. The components in the upper-level system are indicated in the thicker-line box in Figure 4, which has been discussed in the previous section. The lower-level system, shown in the thinner-line box in Figure 4, contains a Dell computer. An image acquisition board is housed in the computer to digitize the camera signal from the Quantix camera. A data acquisition board is also housed in the computer to control the arc lamp shutter (placed in front of Zeiss FluoArc lamp) through a shutter driver. The dual-view system in front of the Quantix camera images two spectrally separated copies of the specimen plane onto the camera. The color channels are aligned before each experiment.

The real-time sperm tracking, automatic laser trapping, and ratiometric fluorescent image processing is custom coded in the LabView language. The operation of real-time tracking and automatic laser trapping is carried out in the upper-level system. Fluorescent image acquisition, processing, and storage are done in the lower-level system. The two computers are networked together over a gigabit TCP/IP cat5e crossover connection (not shown in Figure 4). Communication between the two systems is optimized with LabView's shared variables and VI server functions in which the lower-level system continuously polls the upper-level system for the next request.
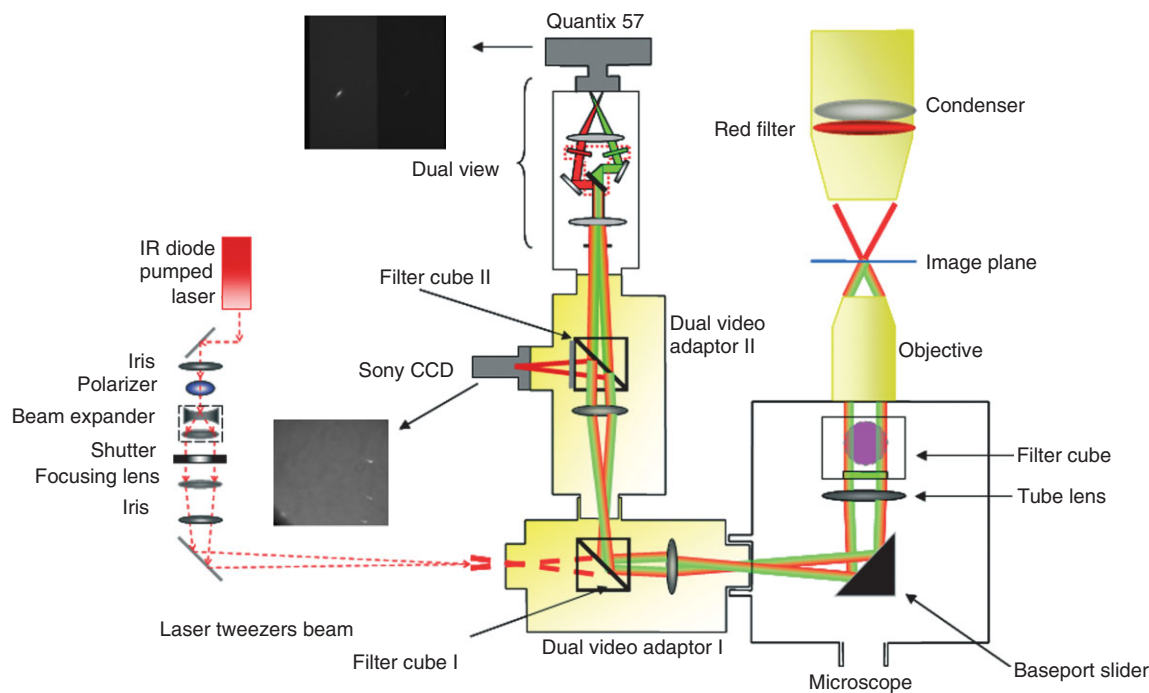

FIGURE 3 | Optical-setup for track, trap and fluorescent ratio measurements.
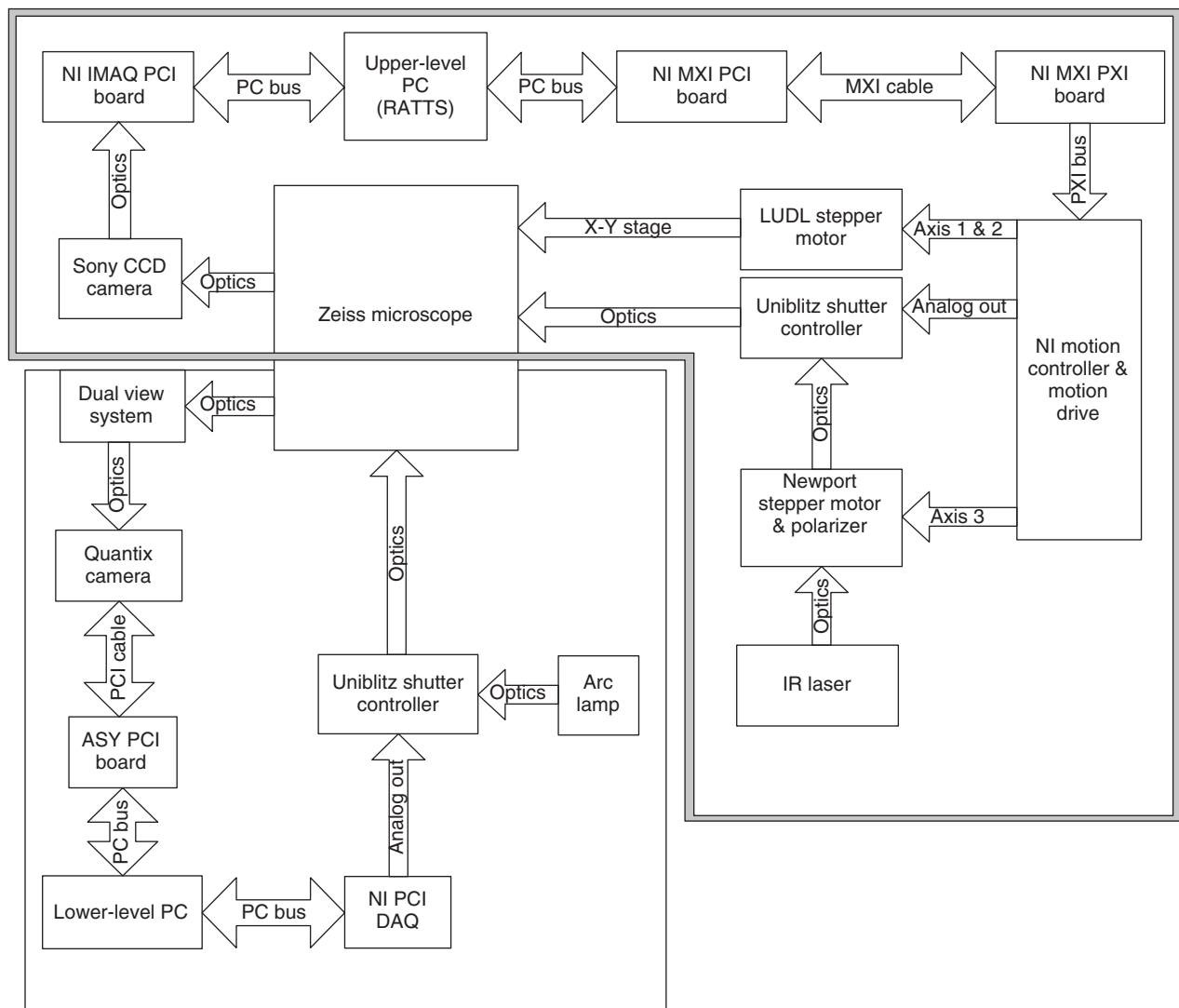
FIGURE 4 | Hardware diagram of the system to study sperm motility and energetics.[17] The upper-level system includes the components in the double-line box, and the lower-level system includes the components in the single-line box. (Reprinted with permission from Ref 17. Copyright 2008 Springer Science+Business Media).

This polling method requires a minimal time for communication. The flow chart of the algorithm for track–trap-fluorescent imaging is shown in Figure 5.[17] The two dashed boxes represent the subroutines in the lower-level computer.

Roles of Oxidative Phosphorylation and Glycolysis

The system of laser tweezers, fluorescent imaging, and RATTS is used to study the roles of two sources of ATP in sperm motility: oxidative phosphorylation, which occurs in the mitochondria located in the sperm midpiece, and glycolysis, which occurs along the length of the sperm tail (flagellum). No relationships

are found between swimming speed and MMP or between swimming force and MMP in dog (total of 309) and human (total of 255) sperm.[18]

In human sperm, the effects of different concentrations of glucose on sperm motility and MMP were determined. Then, the effects of three different inhibitors on sperm motility and MMP were assessed. 2-deoxy-D-glucose (DOG), an antimetabolite of glucose, was used to inhibit the glycolytic pathway.[19] The DOG experiment was repeated three times to verify the results. Data from the three experiments were subsequently pooled together. Antimycin A was used to inhibit the oxidative phosphorylation pathway via the electron transport chain (inhibits the oxidation of ubiquinol in complex III).[17–18] Antimycin A was
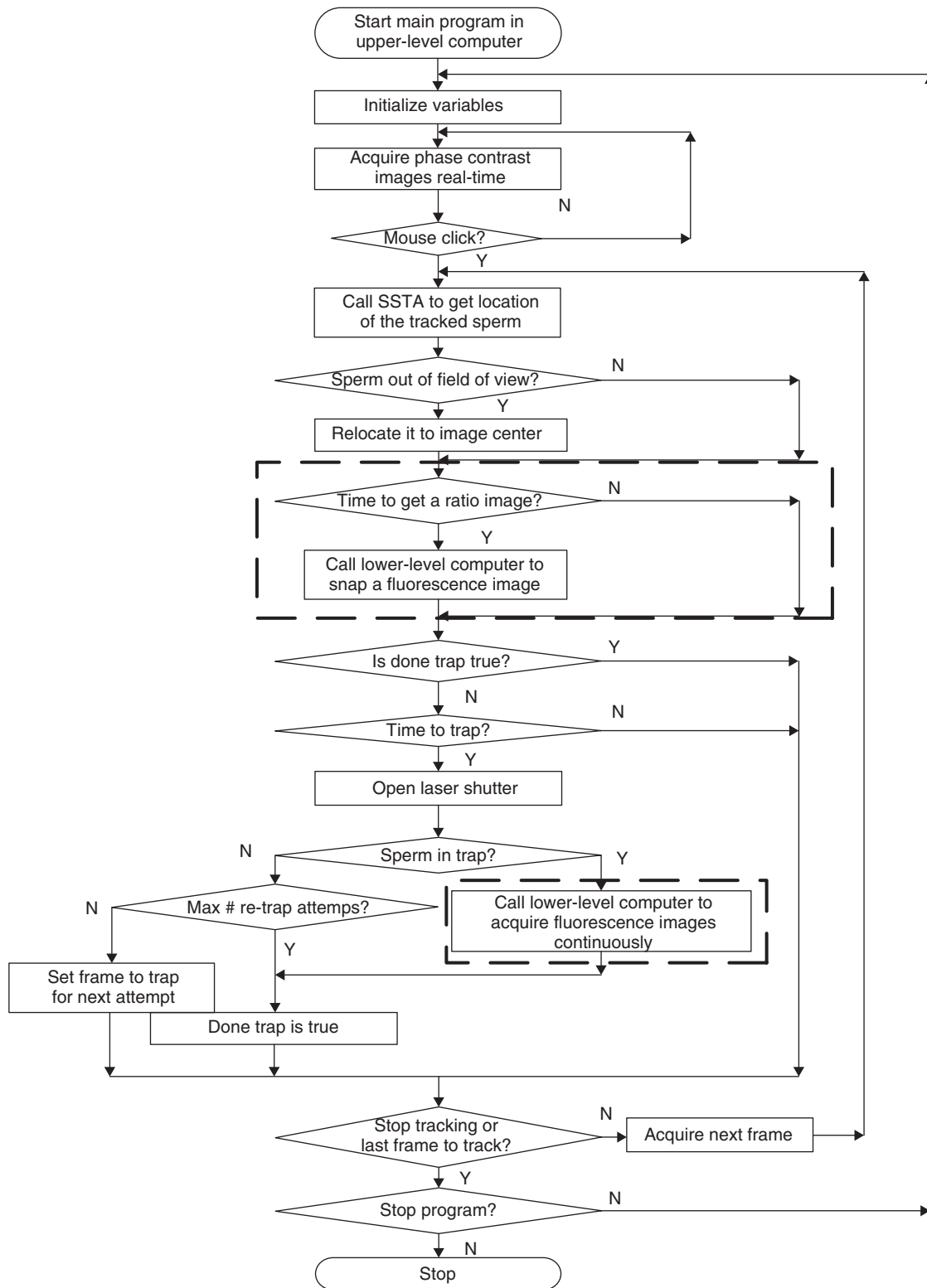
FIGURE 5 | Flowchart of track–trap-fluorescent imaging.[17] The dashed boxes are for the logic of fluorescent imaging. During the experiment, the user selects a sperm to be analyzed by clicking on its image with the arrow cursor on the front panel of real-time automated tracking and trapping system. The cursor coordinate is registered, passed to the tracking algorithm, and computation proceeds with no further intervention. Once the specified number of frames has been processed, the stage is moved to place the sperm under the laser trap and the shutter is opened. (Reprinted with permission from Ref 17. Copyright 2008 Springer Science+Business Media).

chosen for preliminary studies working with human sperm suspended in human tubal fluid (HTF) media.

The effects of glucose, oxidative phosphorylation inhibitors, and glycolytic inhibitors on human sperm motility were examined (Figure 6). The effects of glycolytic and oxidative phosphorylation inhibitors on VCL, Pesc, and MMP in human sperm were described.

1. For DOG, the velocity, escape power, and MMP ratio value (pre-trap) in three different sperm groups were statistically compared using the nonparametric Wilcoxon paired-sample test. Distributions for VCL, Pesc, and MMP are shown in Figure 6(a)–(c), respectively, as the red 'DOG' set of data. Sperm in glucose-positive BWW media even in the presence of DOG were found to be both faster and stronger than sperm from either the glucose-negative control or the glucose-negative with DOG groups. Sperm from the glucose-negative control group and sperm from the glucose-negative with DOG group were found to be statistically equal in terms of VCL and Pesc ($P > 0.05$). All three groups were found to have equal MMP distributions ($P > 0.05$). Therefore, the presence of DOG did not affect sperm mitochondrial MMP.

2. For antimycin A, human sperm in the glucose-negative media exposed to antimycin A were found to be immotile (marked as 'X' in Figure 6(a)–(c)) and therefore were not measured. The velocity, escape power, and MMP ratio value (pre-trap) distributions of the human sperm in the other three groups were statistically compared using the nonparametric Wilcoxon paired-sample test. Distributions for VCL, Pesc, and MMP are shown in Figure 6(a)–(c), respectively, as the black 'Antimycin A' set of data. The velocity distributions of sperm in glucose-positive media with antimycin A were found to be statistically equal ($P > 0.05$). In addition, they were found to be faster than sperm from the glucose-negative control group. For the Pesc distributions, HTF media appeared to be a better buffer against the adverse effects of antimycin A than BWW with glucose. However, sperm in the glucose-positive media with antimycin A were found to be statistically stronger than sperm in the glucose-negative media without the inhibitor. Sperm in the HTF media with antimycin A were found to have a statistically greater median MMP value than the sperm in either of the other two groups. Sperm in the glucose-negative control media had a greater median MMP value

than the sperm in the BWW glucose-positive media with antimycin A. Again, HTF was a better buffer against the effects of the inhibitor than BWW.

3. For rotenone, human sperm in the glucose-negative media exposed to rotenone were found to be immotile (marked as 'X' in Figure 6(a)–(c)) and therefore were not measured. The velocity, escape power, and MMP ratio value (pre-trap) distributions of the human sperm in the other three groups were statistically compared using the nonparametric Wilcoxon paired-sample test. Distributions for VCL, Pesc, and MMP are shown in Figure 6(a)–(c), respectively, as the green 'Rotenone' set of data. The velocity distributions of sperm in glucose-positive media with rotenone were found to be statistically equal ($P > 0.05$). In addition, they were found to be faster than sperm from glucose-negative control group. For the Pesc distributions, HTF media appeared to be a better buffer against the adverse effects of rotenone than BWW with glucose. However, sperm in the glucose-positive media with rotenone were found to be statistically stronger swimmers (Figure 6(a)) than sperm in the glucose-negative media without the inhibitor. The MMP of sperm in the HTF media with rotenone was found to be statistically the same as that of the sperm in the glucose-negative control media ($P > 0.05$). The MMP of sperm in both these groups was statistically greater than that of sperm in the glucose-positive BWW media with rotenone. Again, HTF appeared to be a better buffer against the effects of the inhibitor than BWW.

The results of these rather complex set of studies demonstrated that oxidative phosphorylation did contribute some ATP to human sperm motility, but not enough to sustain high motility. The glycolytic pathway, on the other hand, was shown to be a primary source of energy for human sperm motility. These results were supported in a recent review article[20] that concluded that for sperm motility from a different mammalian species '...both mitochondrial oxidative phosphorylation, for which oxygen is friend, and glycolysis, for which sugar is friend, can provide the energy, independent of one another'. However, in the studies we have discussed here, this has been proven with a sperm-by-sperm analysis approach using an array of optical and computer-based technologies. This approach can now be used to examine sperm energetics across a broad phylogenetic spectrum including vertebrates and invertebrates and
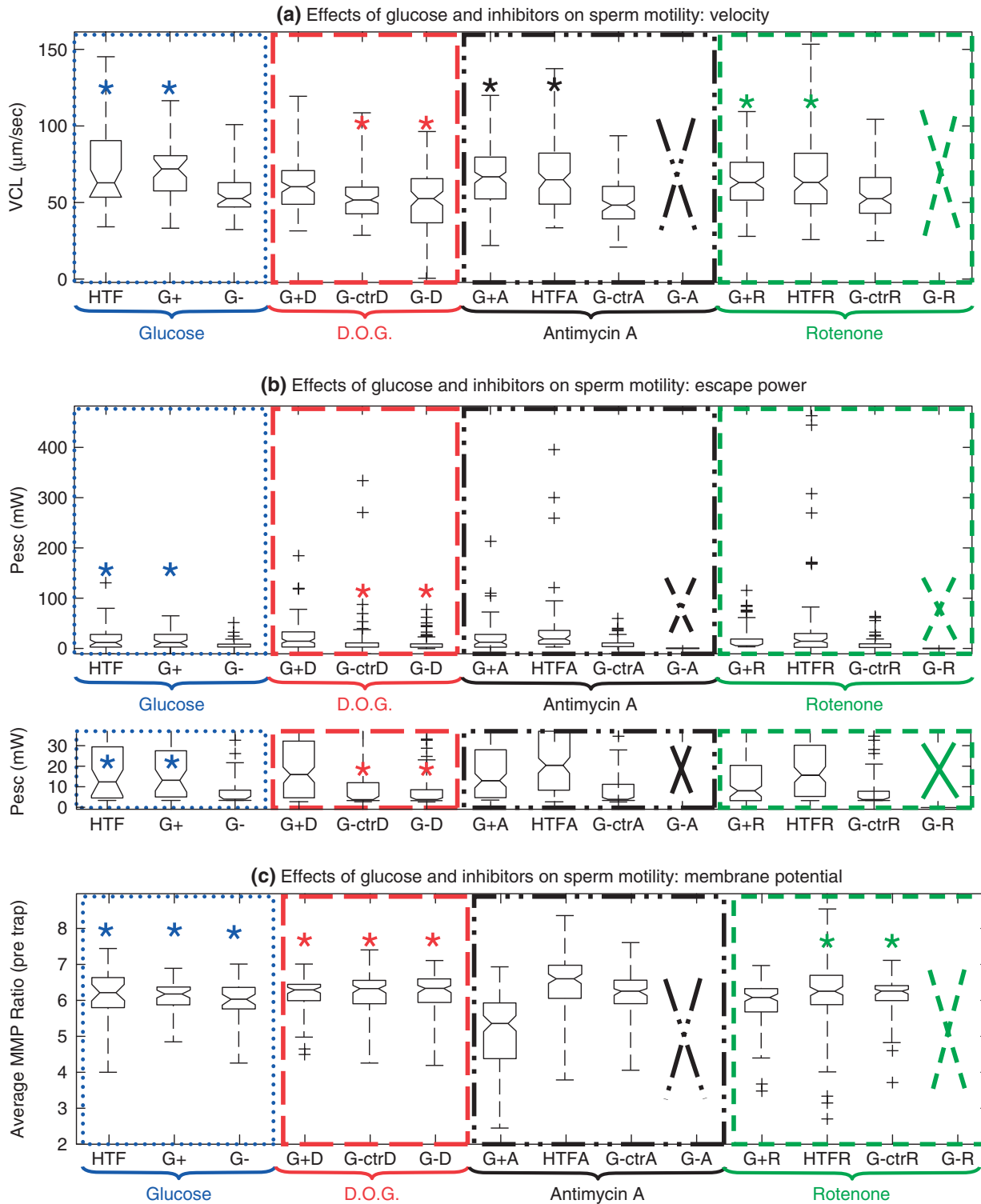
FIGURE 6 | Effects of glucose (blue), 2-deoxy-ᴅ-glucose (DOG; red), antimycin A (black), and Rotenone (green) on the human sperm (a) swimming speed (VCL), (b) swimming force (Pesc), and (c) mitochondrial membrane potential. Stars (*) within each category indicate statistically equal distributions. The Pesc distributions in (b) are shown twice: the top image displays the full escape power range, whereas the bottom image magnifies the box plots to emphasize differences in median values. HTF, human tubal fluid media with glucose; G+, BWW + BSA media with glucose; G−, BWW + BSA media without glucose; ctr, glucose-negative control group (without inhibitor); D, DOG; A, antimycin A; R, rotenone.[18](Reprinted with permission from Ref 18. Copyright 2008 John Wiley & Sons, Inc.).

external versus internal fertilizers. Finally, the role of the female reproductive tract in facilitating or inhibiting the movement of sperm also comes into play, and this will vary between species as well. Thus, the balance between the energetic pathways that have evolved for sperm from different species may indeed be quite variable.

SPERM COMPETITION: A QUESTION OF EVOLUTION

Perhaps one of the most intriguing interdisciplinary applications of optical trapping and computer-based robotics has been to examine the question of sperm competition. RATTS was used to study the question of sperm competition in relation to the mating patterns of different primate species. In species where the mating pattern is polygamous (multimale–multifemale), several different males copulate with a single female within a short time frame (actually minutes). As a result, strong competition for fertilization between the sperm from the rival males occurs within the female reproductive tract.[21] It was theorized that this competition should result in faster and stronger swimming sperm in the polygamous species as opposed to the monogamous species. A single-point laser trap in combination with RATTS was used to test this hypothesis by comparing the swimming force and velocity of sperm from chimpanzees, rhesus macaque, gorilla, and human. The mating systems for both the chimpanzee and the rhesus macaque are multimale–multifemale (females of these species mate with more than one male within a short period of time). Gorillas are polygynous, one male–multiple females, and human mating patterns are variable,

differing across cultures but can be considered to be predominantly polygynous (83% of societies), more rarely monogamous (16%), and only very occasionally polyandrous (<1%[21]). Therefore, the sperm analyzed in this study came from primates that represented a variety of mating patterns, ranging from strictly polygynous (gorilla) to multimale–multifemale (chimpanzee and rhesus macaque).

Figure 7 shows the box plots for distributions of (a) sperm swimming speed (VCL, μm/second) and (b) sperm escape power (Pesc, mW) for the four primates.[22] The inset in Figure 7(b) is an expanded view of the human and gorilla Pesc box plots to emphasize the difference in medians between the two species. Data points lying outside three times the interquartile range are present only in the swimming force distributions. These 'outliers' represent a small percentage of the sperm population (2.57% of chimpanzee population, 0% of rhesus macaque population, 3.46% of human population, and 7.69% of gorilla population). The swimming speed and escape force distributions of each species are found to be statistically different ($P < 0.05$) using the Wilcoxon rank-sum test. The medians of both measurements, VCL and Pesc, show that rhesus macaque and chimpanzee sperm swim with the fastest speeds and the strongest forces, whereas gorilla sperm swim with the slowest speeds and weakest forces. Human sperm swimming speeds and swimming forces lie between these two extremes. The data demonstrate that escape force and swimming speed increase as the level of sperm competition increases. These results support the theory that sperm from primates that are polygamous have experienced high competitive pressures and thus have evolved to swim both stronger and faster than sperm
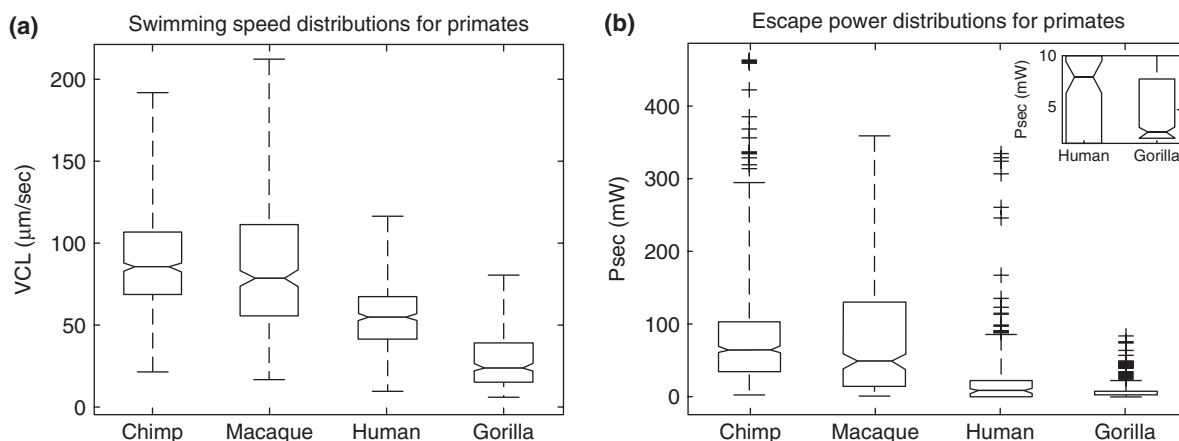

FIGURE 7 | Swimming speed and escape power distributions.[22] Box plots of the distributions of (a) swimming speed (VCL, Em/s) and (b) escape power (Pesc, mW) for all four primates. Inset in (b) shows a magnified view of human and gorilla. (Reprinted with permission from Ref 22. Copyright 2008 The Royal Society).

from primates that have not been under such high competitive pressures. In summary, laser trapping and the RATTS computer/robotic systems were applied to an important and intriguing evolutionary question. This is a good example of interdisciplinary approach to systems research.

FUTURE STUDIES

In this article we have reviewed studies that have been conducted over a 20-year period that demonstrate how engineering approaches, such as optical

manipulation, computer-based robotic control of systems, and image-based analysis and computation, can be combined to provide a powerful interdisciplinary approach to study the complex biological systems that drive the sperm toward the egg. It is anticipated that this combined interdisciplinary approach to biological systems will become even more widespread over the coming decades. Not only will this lead to a better understanding of the systems studied (in this case the role of sperm energetics in fertilization), but also these same engineering technologies, as well as others that are still in early stages of development, will be applied to an even wider array of biological problems.

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