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Optical tweezers and non-ratiometric fluorescent-dye-based studies of respiration in sperm mitochondria

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Abstract
The purpose of this study is to investigate how the mitochondrial membrane potential affects sperm motility using laser tweezers and a non-ratiometric fluorescent probe, $DiOC_6(3)$. A 1064 nm Nd:YVO4 continuous wave laser was used to trap motile sperm at a power of 450 mW in the trap spot. Using customized tracking software, the curvilinear velocity (VCL) and the escape force from the laser tweezers were measured. Human (*Homo sapiens*), dog *(Canis lupis familiaris*) and drill (*Mandrillus leucophaeus)* sperm were treated with $DiOC_6(3)$ to measure the membrane potential in the mitochondria-rich sperm midpieces. Sperm from all three species exhibited an increase in fluorescence when treated with the $DiOC_6(3)$. When a cyanide inhibitor (CCCP) of aerobic respiration was applied, sperm of all three species exhibited a reduction in fluorescence to pre-dye levels. With respect to VCL and escape force, the CCCP had no effect on dog or human sperm, suggesting a major reliance upon anaerobic respiration (glycolysis) for ATP in these two species. Based on the preliminary study on drill sperm, CCCP caused a drop in the VCL, suggesting potential reliance on both glycolysis and aerobic respiration for motility. The results demonstrate that optical trapping in combination with $DiOC_6(3)$ is an effective way to study sperm motility and energetics.

Keywords: sperm motility, sperm energetics, optical tweezers, non-ratiometric fluorescent dye

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Sperm motility is a valuable marker of sperm quality and a good indicator of the potential for the sperm to eventually fertilize an egg. The ability to evaluate sperm motility is critical for human *in vitro* fertilization (IVF), and when trying to improve the reproduction of rare and endangered species. Successful insemination requires high quality sperm. In addition, the ability to quantify and analyze sperm motility is crucial when developing cryopreservation techniques, which are widely used in *in vitro* fertilization and reproductive biology research (Wolf and Patton 1989, Mahutte and Arici 2003).

The basic sperm structure consists of a head, midpiece and tail. The head has very little cytoplasm and minimal organelles (Schmidt-Rhaesa 2007), favoring a dense amount of genetic material and the acrosome. The acrosome, which tends to be either at the tip or wrapped around the head, contains enzymes to help the sperm penetrate the egg. The midpiece sits below the head and is the site of mitochondrial aerobic respiration and supplies ATP for motility and other functions. The size of the midpiece and amount of mitochondria varies greatly between different species, reflecting different needs with regard to fertilization (Favard and Andre 1970). The tail, or flagellum, provides the propulsion for movement.

Curvilinear velocity (VCL) is a well-established parameter for comparison of sperm motility between or within species. It is generally measured in large populations using computer-assisted sperm analysis (CASA). In addition to VCL, laser

trapping has been used to measure the actual swimming force of human sperm (Tadir *et al* 1989) as well as sperm from other vertebrate species (Nascimento *et al* 2006). The ideal optical tweezer wavelengths for sperm trapping fall between 800 and 1064 nm (Konig *et al* 1996). Using laser tweezers, Patrizio found that the swimming force of sperm dramatically increases upon addition of pentoxifylline (Patrizio *et al* 2000), while Nascimento studied sperm competition based on mating type (polygamous versus monogamous) in several primate species, including human (Nascimento *et al* 2008a). In addition, the contribution of anaerobic (glycolysis) versus aerobic (oxidative phosphorylation) respiration has been studied using a ratiometric membrane potential dye $DiOC_2(3)$ (Nascimento *et al* 2008b).

$DiOC_6(3)$, on the other hand, is a non-ratiometric carbocyanine dye that has the potential to be used to investigate sperm motility and energetics. This dye has been found to successfully integrate into mouse sperm (Breed and Sarafis 1995), the endoplasmic reticulum, mitochondria (Sabnis *et al* 1997) and human sperm (Gallon *et al* 2006). It is a positively charged molecule that targets the negative membrane potential of the mitochondria, resulting in a greater fluorescence when the membrane potential is larger (Wang and Taylor 1989). Inhibition of oxidative phosphorylation (aerobic respiration) can be achieved with a variety of substances. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) is an inhibitor that works by disrupting the mitochondrial membrane potential and discharging the H+ gradient in the mitochondria (Alvarado and Vasseur 1998). Successful integration of CCCP into the cell, and subsequently the mitochondria, would directly attack the source of $DiOC_6(3)$ fluorescence, resulting in a decrease or complete inhibition of fluorescence. In this study we demonstrate that this dye in combination with optical trapping can be used to study sperm motility and energetics.

2. Material and methods

2.1. Sample collection and preparation

Semen samples for human (*Homo sapiens*) and dog (*Canis lupis familiaris*) were obtained and handled as previously described (Nascimento *et al* 2008a, Nascimento *et al* 2008b). Drill (*Mandrillus leucophaeus*) samples were collected weekly and cryopreserved following a protocol similar to dog (Durrant *et al* 1999). The samples were thawed and centrifuged at 2200 rpm for 10 min and decanted. The pellet was mixed with Medium 199 (M199), and ∼50 μl of sperm are diluted into 2.5 ml media and loaded into rose culture chambers. For all three species, the sperm were incubated in CCCP at 10 nM for 20 min before undergoing testing.

2.2. Optical set-up and real-time automated tracking and trapping system (RATTS)

The tracking and trapping system uses an Nd:YVO4 continuous wave 1064 nm wavelength laser traveling through a series of lenses and mirrors to enter a Zeiss Axiovert S100 microscope and a 40×, phase III, NA 1.3 oil immersion objective as described previously (Nascimento *et al* 2008a, 2008b). The maximum laser power after traveling through the microscope and objective lens is approximately 450 mW at the focal point. This was determined by measuring the amount of laser power at the back aperture of the microscope objective and multiplying that by the transmission (33%) through the objective. The objective transmission was determined using the dual-objective method (Misawa *et al* 1991).

The microscope configuration permits collection of phase and fluorescence images (figure 1). The laser passes through a dichroic long-pass filter that reflects visible light from the microscope optical system to the above-mounted camera systems. Before it hits the objective, a filter cube with an HQ 475/25 nm excitation filter and 505 nm dichroic filter permits fluorescence activation of the specimen in the image plane. A Zeiss Fluor arc lamp (not depicted in figure 1) provides the excitation light via epi-illumination through the microscope objective. The red filter above the image plane allows 670 nm wavelength light to pass through the specimen in order to generate the phase contrast image on the Cohu digital camera. The dual video system attached to the top port of the first video adapter separates the phase information (reflects >670 nm) from the fluorescence information (transmits 500–670 nm). The reflected phase image is collected by a Cohu charge-coupled device (CCD) camera at 30 frames s^{-1} (fps). The fluorescence information passes through an HQ 500/20 nm emission filter and is collected by a high sensitivity Quantix digital camera.

Measurement of motility and fluorescence is accomplished with the aid of a real-time automated tracking and trapping system (RATTS) that operates at a 30 fps video rate and provides remote robotic interfaces with the hardware (Shi *et al* 2006). RATTS has been modified to measure the mitochondrial membrane potential (prior to, during and after laser trapping) in conjunction with swimming speed and escape laser power (swimming force) of individual sperm. Using RATTS, sperm are tracked for extended durations before and after laser trapping. 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. The fluorescence was evaluated by taking a small region of interest surrounding the sperm and measuring the intensity. Fluorescence of untreated sperm was considered the baseline. Fluorescence ratio was determined by finding the difference of measured fluorescence intensity of a sperm and the baseline fluorescence, and then dividing by the baseline. A fluorescence value close to or at zero indicates little or no fluorescence, and a value greater than zero indicates noticeable fluorescence. Fluorescence was evaluated during the pre-trap and trapping phase. When fluorescence was tracked for a long period of time (much longer than the program would track and trap a sperm), fluorescence was relatively consistent (data not shown).

3. Results

3.1. Optimal $DiOC_6(3)$ concentration for fluorescence

The purpose of these experiments was to determine the optimal concentration of the $DiOC_6(3)$ dye necessary to detect and

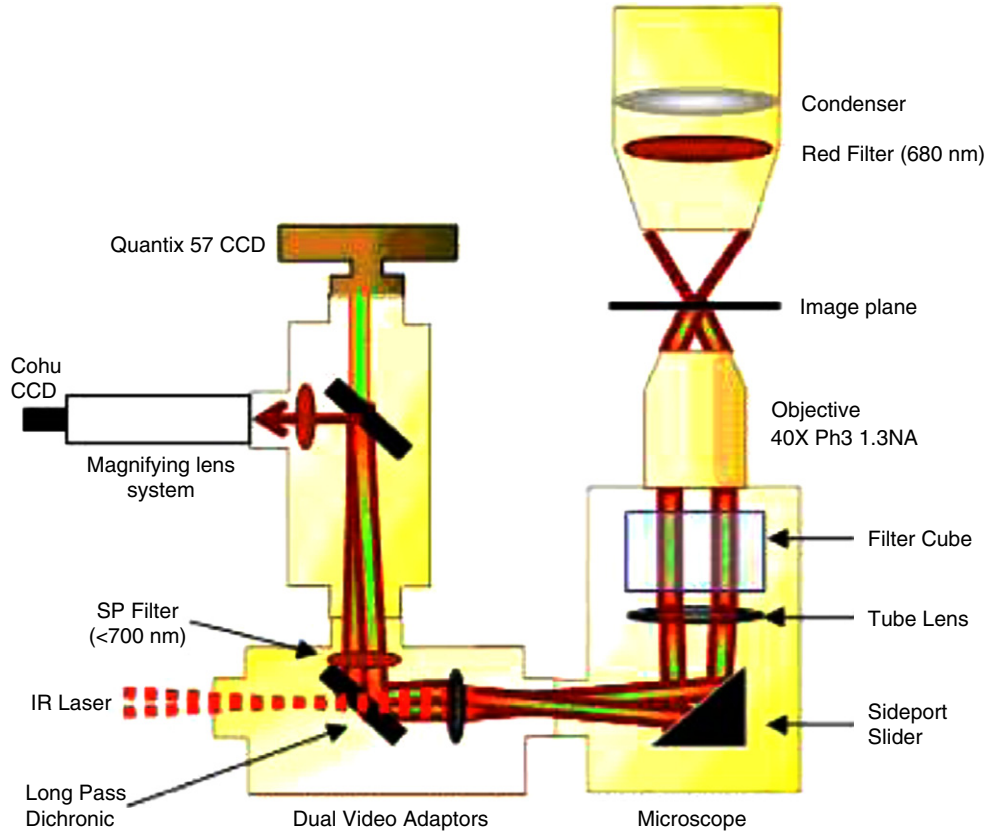

Figure 1. Optical system: the laser travels to the image plane, while the fluorescence image passes through a dichroic beamsplitter in the dual video adapter to the low light level Quantix CCD camera and the phase image is reflected to the Cohu digital camera.

Figure 2. $DiOC_6(3)$ midpiece fluorescing in three species (human, dog and drill). The optimal concentration of $DiOC_6(3)$ is 40 nM for human and 50 nM for dog and drill.

measure fluorescence in individual sperm. The fluorescence of the untreated sperm is considered the baseline fluorescence. Fluorescence ratio was determined by finding the difference of measured fluorescence intensity of a sperm and the baseline fluorescence, and then dividing by the baseline. A fluorescence value close to or at zero indicates little or no fluorescence, and a value greater than zero indicates fluorescence. Human sperm were evaluated after exposure to 30, 40 and 50 nM of dye. The dog sperm were exposed to 40 and 50 nM, and the drill to 10, 30 and 50 nM. The optimal concentration of $DiOC_6(3)$ is 40 nM for human and 50 nM for dog and drill (figure 2).

The dye was added after the sperm washing protocols and incubated at 37 °C for 20 min. Using the above fluorescence algorithm, fluorescence values were determined at the different concentrations. The further the value from zero, the greater the fluorescence. Human had fluorescence of 0.25, 0.23 and 0.16 at 30, 40 and 50 nM, respectively. Dog values were 0.23 and 0.21 at 40 and 50 nM. Drill fluorescence values were 0.03, 0.08 and 0.3 at 10, 30 and 50 nM. The possible reason for the drop in fluorescence of human sperm could be due to excess $DiOC_6(3)$ disrupting respiration, thus affecting the membrane potential (Wang and Taylor 1989). This allows the dye to interact with other membranes, such as the endoplasmic reticulum, which the dye is known to stain at higher concentration. The nonlinear staining could be due to natural variability of the dye (Wang and Taylor 1989) or an insufficient dye quantity to stain the larger drill midpiece. The optimal experimental concentrations chosen were 40 nM for human and 50 nM for

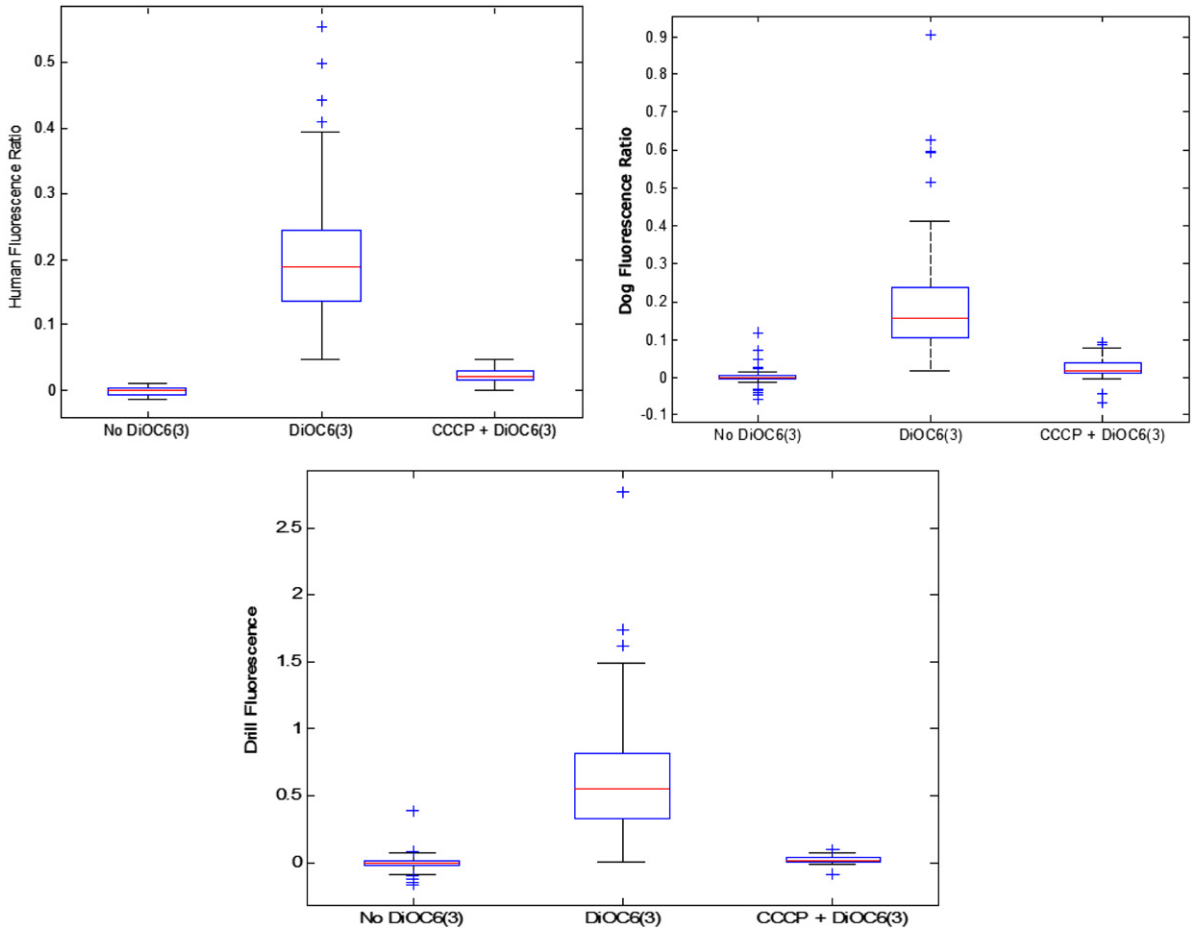

Figure 3. Box plots for human, dog and drill sperm. Each species showed an increase in fluorescence upon addition of $DiOC_6(3)$ and a drop in fluorescence after CCCP was added. The center of the box represents the median, and the upper and lower edge of the boxes represents the upper and lower quartiles of the data. The outer edge of the bars represent data to the ± 2.7 standard deviations, thus covering 99.3% of data if normally distributed. The crosses represent outliers.

dog and drill, which were the concentrations used in further testing.

3.2. Motility and energetics measurements

For each of the species, three different groups were measured: (1) sperm incubated in the fluorescent membrane probe $DiOC_6(3)$; (2) sperm incubated in $DiOC_6(3)$ plus a cyanide inhibitor of oxidative phosphorylation (CCCP), subsequently referred to as 'dye + CCCP-treated' and (3) untreated control sperm. Since sperm populations do not have Gaussian distributions with respect to motility determinants (Nascimento *et al* 2008a), the non-parametric Wilcoxon rank sum test was used for statistical analyses (Donnelly *et al* 2001). Each of the three groups was compared as follows: untreated versus dye; untreated versus dye + CCCP-treated; dye versus dye + CCCP-treated. Fluorescence and VCL were plotted against each other in order to elucidate any relationship between mitochondrial activity and sperm motility.

When $DiOC_6(3)$ was added, all three species showed a significant increase in fluorescence over controls (figure 3). The addition of the CCCP inhibitor caused a decrease to the control levels. With respect to the VCL and swimming force measurements (table 1), for dog and human sperm there was no apparent decrease in either the VCL or swimming force when the dye or the dye + CCCP were added to the medium. The swimming force and VCL for dog sperm were higher than for human sperm. The data for fluorescence versus velocity is presented in figure 4. For the drill sperm, the VCL appeared to decrease slightly for the dye + CCCP-treated sperm. The p value between the untreated sperm versus dye-and CCCP-treated sperm was 8.27×10^{-11}, which is statistically significant. Table 2 contains the p values for other group comparisons. The swimming force seemed to increase in the dye + CCCP-treated group when compared to the control.

4. Discussion

The results of this study demonstrate that the non-dual-wavelength ratiometric dye $DiOC_6(3)$ is effective in monitoring the mitochondrial membrane potential (aerobic respiration) in motile sperm. The addition of the aerobic respiration inhibitor, CCCP, eliminated fluorescence in all three species while having no inhibitory effect on swimming forces and VCL in both the dog and human. If aerobic respiration is the primary energy source for motility in these two species,

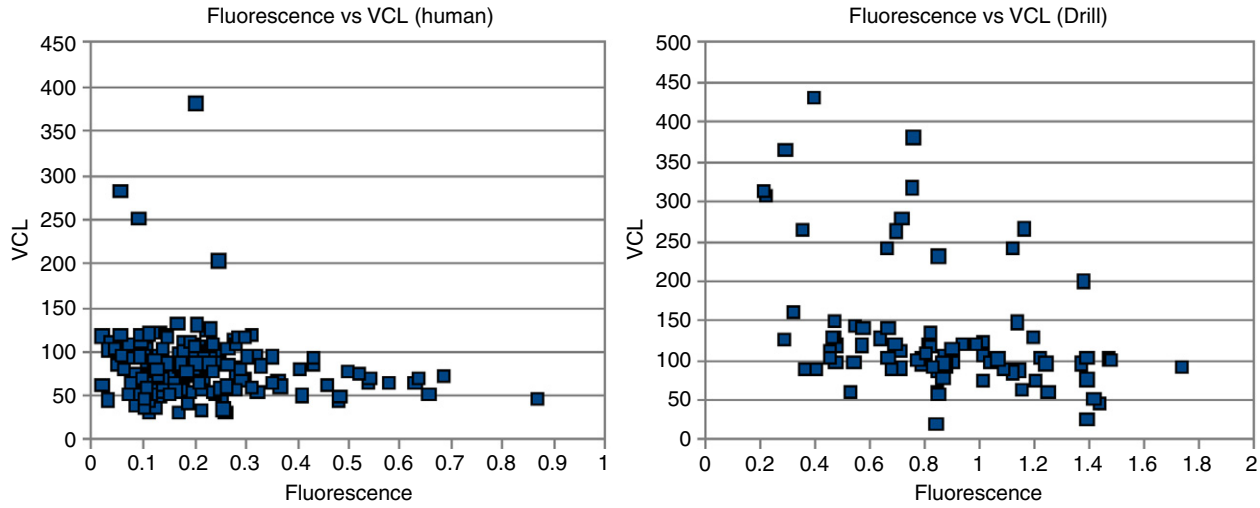

Figure 4. Fluorescence ratio versus VCL in human and drill sperm. There appears to be no correlation between fluorescence and VCL.

Table 1. The means of the VCL, swimming force and fluorescence for untreated sperm (no dye), dye-treated sperm and sperm with CCCP and dye.

	No dye	Dye	CCCP + dye
Human VCL (μm s^{-1})	82.13 ($n = 148$)	84.58 ($n = 411$)	82.65 ($n = 160$)
Human swimming force (pN)	38.1 ($n = 114$)	26.2 ($n = 251$)	31.0 ($n = 87$)
Human fluorescence ratio	-1.74×10^{-5} ($n = 65$)	0.202 ($n = 122$)	0.023 ($n = 78$)
Dog VCL (μm s^{-1})	108.97 ($n = 334$)	106.44 ($n = 472$)	108.77 ($n = 370$)
Dog swimming force (pN)	42.0 ($n = 104$)	46.6 ($n = 116$)	50.1 ($n = 54$)
Dog fluorescence ratio	-3.65×10^{-6} ($n = 100$)	0.192 ($n = 103$)	0.025 ($n = 53$)
Drill VCL (μm s^{-1})	113.56 ($n = 256$)	117.42 ($n = 280$)	96.11 ($n = 162$)
Drill swimming force (pN)	44.5 ($n = 17$)	39.2 ($n = 16$)	55.5 ($n = 35$)
Drill fluorescence ratio	-1.57×10^{-5} ($n = 277$)	0.62 ($n = 273$)	2.69×10^{-2} ($n = 137$)

Table 2. The p values from the Wilcoxon rank sum test. Values below 0.05 are considered statistically significant, and are in bold. The first column (no dye versus dye) compares untreated sperm versus $DiOC_6(3)$-treated sperm. The second column compares untreated sperm and sperm treated with $DiOC_6(3)$ and CCCP. The final column compares sperm treated with dye and sperm treated with the dye and CCCP.

p values from the Wilcoxon rank sum test	No dye versus dye	No dye versus CCCP + dye	Dye versus CCCP + dye
Human VCL	0.93	0.65	0.62
Human swimming force	0.34	0.06	0.32
Human fluorescence ratio	$\mathbf{2.37 \times 10^{-29}}$	$\mathbf{6.16 \times 10^{-22}}$	$\mathbf{9.85 \times 10^{-33}}$
Dog VCL	0.06	0.18	0.33
Dog swimming force	0.39	0.27	0.73
Dog fluorescence ratio	$\mathbf{5.19 \times 10^{-34}}$	$\mathbf{9.33 \times 10^{-15}}$	$\mathbf{2.20 \times 10^{-21}}$
Drill VCL	0.15	$\mathbf{8.27 \times 10^{-11}}$	$\mathbf{1.92 \times 10^{-8}}$
Drill swimming force	0.44	0.47	0.96
Drill fluorescence ratio	**0**	**0**	$\mathbf{2.69 \times 10^{-60}}$

it would be expected that the VCL would drop considerably when the CCCP is added. The results of previous studies on mammalian motility (Nascimento *et al* 2008b, Storey 2008) and the results reported here with the CCCP inhibitor demonstrate that glycolysis is a primary source of the ATP for sperm motility in human and dog.

The results with the drill sperm are less clear. The decrease in VCL in the dye + CCCP group indicates a reliance on aerobic respiration for ATP. However, the increase in swimming force for this group is perplexing. It is possible that the dye may be causing sperm hyperactivation resulting in greater lateral head movements with a stronger swimming force and at the same time a reduction in the VCL. The higher signal-to-noise ratio may indicate a difference in dye uptake, or more activity within the midpiece, and thus a greater membrane potential. Evolutionarily, human and dog are not conventionally seen as competitive reproductively within their own species. Wild dogs travel in packs of only 4–8 adults (Woodroffe and Ginsberg 1999) and humans are closer to a mating system with lower sperm competition (Martin 2007). Humans have some of the smallest midpieces amongst primates, indicating that activity in the midpieces is not nearly as important for sperm motility as in other species with larger midpieces. Drill has a larger midpiece volume (Anderson *et al* 2005), which has been correlated with multi-male systems (Anderson and Dixson 2002), possibly due to the evolution of higher energy requirements because sperm from different males are competing to get to the egg. However, the sample size for the drill was smaller compared to the other species and the data, therefore, should be viewed as preliminary.

In conclusion, the results reported here demonstrate that optical trapping in combination with the non-ratiometric dye $DiOC_6(3)$ are effective for the study of sperm motility and energetics in both common species (dog and human), and in rare and highly endangered species, such as the drill.

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