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A Real-time Single Sperm Tracking, Laser Trapping and Ratiometric Fluorescent Imaging System

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ABSTRACT

Sperm cells from a domestic dog were treated with oxacarbocyanine DiOC₂(3), a ratiometrically-encoded membrane potential fluorescent probe in order to monitor the mitochondria stored in an individual sperm's midpiece. This dye normally emits a red fluorescence near 610 nm as well as a green fluorescence near 515 nm. The ratio of red to green fluorescence provides a substantially accurate and precise measurement of sperm midpiece membrane potential. A two-level computer system has been developed to quantify the motility and energetics of sperm using video rate tracking, automated laser trapping (done by the upper-level system) and fluorescent imaging (done by the lower-level system). The communication between these two systems is achieved by a networked gigabit TCP/IP cat5e crossover connection. This allows for the curvilinear velocity (VCL) and ratio of the red to green fluorescent images of individual sperm to be written to the hard drive at video rates. This two-level automatic system has increased experimental throughput over our previous single-level system (Mei et al., 2005) by an order of magnitude.

Keywords: Single Sperm Tracking, Laser Trapping, Fluorescent Imaging, Membrane Potential Fluorescent Probe

1. INTRODUCTION

Fluorescent measurements in single-cell analyses allow for the understanding and quantification of a cell's physiological characteristics such as its enzymatic activity and membrane potential (Shapiro, 2000). In particular, fluorescent techniques used to measure membrane potentials have become promising tools for the analysis of sperm functionality. This has been demonstrated in past studies on sperm metabolism by studying the relationship between mitochondrial respiration and sperm vitality (Mei, et al. 2005) and quality (Marchetti, et al., 2002). Kasai, et al. (2004) proposed that sperm motility was dependent on mitochondrial function, and as the sperm mitochondrial membrane potential increased, sperm motility parameters also increased together with their fertility potential. Concurrently in 2004, Martinez-Pastor, et al. indicated that good sperm motility and high velocity values were associated with high mitochondrial membrane potential.

In order to quantify membrane energetics, a variety of dyes have been used in the literature. The fluorescent dye DiOC₆3 was used in Marchetti, et al. (2002), rhodamine 123 (R123) in Paniagua-Chavez, et al. (2006), JC-1 dye (5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) in Troiano, et al. (1998), Kasai, et al. (2004), Martinez-Pastor, et al. (2004), Mei, et al. (2005). In a previous paper using JC-1 dye it was shown that the laser trapping of domestic dog sperm produced a depolarization of the mitochondria, (Mei, et al., 2005). Yet in that experiment it was observed that the overall sperm motility might have been slightly affected after adding JC-1 dye (data was not included). A study by Novo, et al. (1999) on bacterial physiology found that the ratiometric technique for measuring membrane potential using DiOC₂(3) was substantially more accurate and precise than JC-1. Given the possible negative effects of JC-1 and the success of DiOC₂(3) in bacteria, DiOC₂(3) was chosen as the fluorescent probe. Further analysis proved that this dye has no-effect on overall sperm motility (data was not included).

Along with monitoring sperm energetics, investigators have also been interested in quantifying sperm motility. They have achieved this through the use of Computer Assisted Sperm Analysis (CASA) systems. These systems have been commercially available since the mid-1980s and a detailed review of CASA can be found in Amann, et al. (2004) and Mortimer (1994). In order to further speed up the automated measurable parameters of sperm motility, Young, et al.

(1996) and Kuo et al. (2000) developed a real-time automated-stage sperm tracking system (ASTS) to analyze highly dilute sperm suspensions at 30 frames/second.

An alternative approach to measuring sperm motility is to use optical tweezers (traps) to measure sperm swimming forces. Effects of optical trapping on sperm motility have been well characterized in the discussion of Tadir et al. (1989), Dantas et al. (1995), Konig, et al. (1996), and Nascimento, et al. (2006). In 2006, new advances in hardware and software have allowed for the development of a Real-time Automated Tracking and Trapping System for sperm, RATTs (Shi, et al., 2006) that measured both the force and VCL of individual sperm at video rates. After initially identifying and clicking the computer mouse on the sperm of interest, RATTs performed all further tracking and trapping functions without human intervention.

To facilitate the analysis of both the motility and mid-piece membrane potential of sperm, a two-level integrated system has been developed using real-time “track & trap” (done by upper-level system) and fluorescent imaging (done by lower-level system). In this system phase contrast images are acquired at video rates and digitized for image analysis and display. Once the user selects a sperm with the mouse on the upper-level computer, three subsequent operations are performed automatically: (1) Pre-trap: the selected sperm is continuously tracked for a specified sequence length and the fluorescent images are sampled periodically, (2) In-trap: the sperm is aligned with the laser trap with a constant laser power and the fluorescent images are sampled continuously, (3) Post-trap: The same sperm is continuously tracked and the fluorescent images are sampled periodically.

2. MATERIALS AND METHODS

2.1 Optical system

The optical system was described partially in Nascimento et al. (2006) and Mei, et al. (2005). A Nd:YVO₄ continuous wave 1064 nm wavelength IR laser (BL-106C, Newport Corp., Newport, CA) is focused into a Zeiss Axiovert S100 microscope equipped with a 40x [phase III, NA 1.3] oil immersion objective (Zeiss, Thornwood, NY). The laser creates a single-point 3-D gradient laser trap. The specimen is illuminated with both the halogen lamp for phase images and the arc lamp for fluorescent images. The halogen light is passed through a red filter before entering the sample. The fluorescence excitation light is provided by a Zeiss FluoArc. The fluorescence filter cube contains an HQ 500/20 nm excitation filter and a dichroic beam splitter with a 505 nm cut-on wavelength. A dual video adapter attached to the microscope side port contains a filter cube with a dichroic beam splitter (transmitting to the objective the 1064 nm laser light entering from the side port and reflecting visible light coming from the specimen to the top port). A second dual video adapter is attached to the top port of the first video adapter. The filter cube in this second adapter reflects phase images (>650 nm) and transmits fluorescent images (500-700 nm). The phase contrast images are acquired through a regular CCD camera (XC-75, Sony, Japan). An HQ 675/50 M bandpass filter is placed in front of the CCD to block any back reflections from the IR laser. For the fluorescent images, a Dual-View system (Optical-Insights, Tucson, AZ) splits the red and green fluorescent light emitted by the specimen to produce two identical images. Fluorescent filters are obtained from Chroma (Chroma Technology Corp., Rockingham, VT) and are placed in this emission-splitting system (green fluorescence emitter is an HQ 535/40 nm M filter and red fluorescence emitter is an HQ 605/50 nm M filter). The Dual-View system is coupled to a digital camera (Quantix 57, Roper Scientific Inc., Tucson, AZ) that captures the fluorescent images.

2.2 Sperm preparation with DiOC₂(3) dye

Cryogenically frozen domestic dog sperm were thawed in a water bath (~1 minute) and the cells were pelleted by centrifugation (10 minutes/2000 rpm). The supernatant was removed and the pellet was resuspended in 1mL of Biggers, Whittens, and Whittingham (BWW) and bovine serum albumin (BSA) media. Half of the stock solution is aliquoted for dye preparation. 3 μ L of 10 μ M DiOC₂(3) was added to the 0.5mL stock sperm solution to achieve 30 nM final concentration. The cells were incubated for 15 minutes and centrifuged for 10 minutes/2000 rpm.

The protocol for the MitoProbe assay kit (Invitrogen, Carlsbad, CA) for flow cytometry recommends ‘flicking’ the tube to resuspend the cells. However, it was found for our application, the green channel signal was better if the supernatant was removed and fresh BWW+BSA were added to the dyed cells. This increases the signal to noise ratio in the green channel.

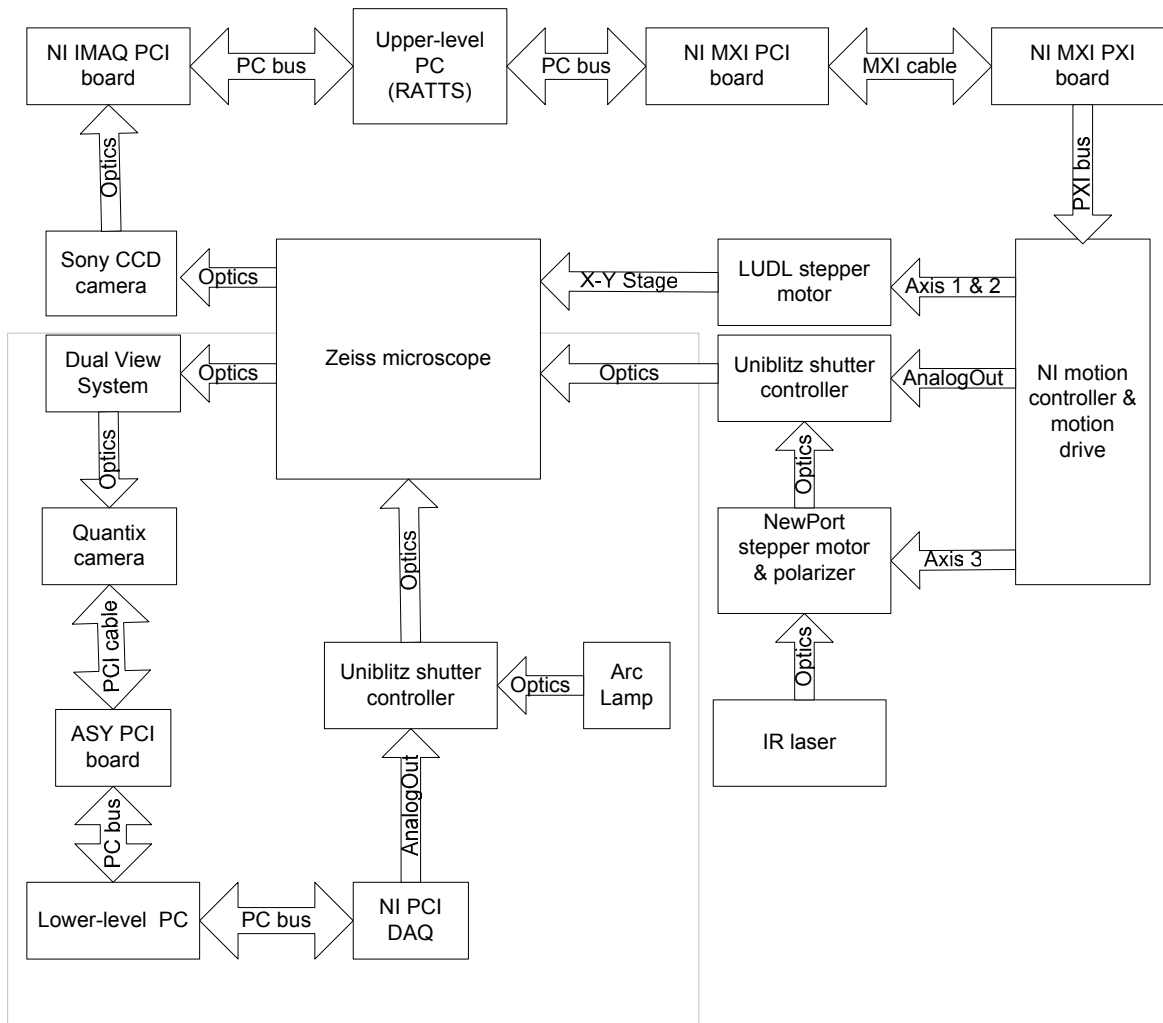


Figure 1 Hardware diagram of real-time single sperm tracking, laser trapping and ratiometric fluorescent imaging system

2.3 System hardware control

Figure 1 shows the system hardware diagram. The hardware setup of the upper-level system can be seen in Shi, et al. (2006). The lower-level system, shown in the gray box in Figure 1, contains a Dell Socket 478 P4 planar mother board supporting an Intel Pentium 4 CPU (2.8GHz). An image acquisition board (Roper Scientific) is housed in the computer to digitize the camera signal from Quantix camera. A DAQ board (NI 6024e, National Instruments, Huston, TX) is also housed in the computer to control the arc lamp shutter (Uniblitz LS6ZM2, Vincent Associates, Rochester, NY) (placed in front of Zeiss FluoArc) through a shutter driver (Uniblitz VMM-D3, Vincent Associates).

2.4 System Software Design

The video rate sperm tracking, automatic laser trapping, and ratiometric fluorescent image processing is custom coded in the LabView 7.1.1 language (National Instruments). The fluorescent image processing is an add-on feature in RATTS. The operation with other features in RATTS is performed in the upper-level system as shown in Shi, et al. (2006). Fluorescent image acquisition, processing and storage are done in the lower-level system. The flow chart of the RATTS algorithm with the fluorescent imaging feature is shown in Figure 2. The two dashed boxes represent the subroutines in the lower-level computer.

The two computers are networked together over a gigabit TCP/IP cat5e crossover connection (not shown in Figure 1). To facilitate the communication between the two systems, Labview VI Server functions are used in which the lower-level system continuously polls the upper-level system for the next request. Unlike other methods, this requires a minimal time for communication. The RATTs on the upper-level system begins its initialization by establishing a one-way connection and autonomously runs the subroutine on the lower-level system. This subroutine then opens up another one-way connection to the RATTs, allowing a two-way cross-communication between the systems. During “track & trap”, the RATTs sets a command variable when it is time to acquire the fluorescent image (the variable is set to 1 for pre-trap, 2 for post trap, and 3 for in-trap). The subroutine snaps a fluorescent image if the command variable changes to 1 or 2, and continues acquiring the images when the command variable is set to 3.

The Dual-View system in front of the Quantix camera has to be aligned by the custom designed Labview code prior to each experiment. The full image acquired from the Quantix camera is first split into its left and right (red and green) halves. The two channels are aligned using the grid (provided by Optical-Insights) to properly display a ratiometric image. A region of interest is used for both channels. One channel’s ROI remains stationary while the other’s ROI shifts by [-5:5] rows and columns to find the highest correlation of the two ROIs.

The subroutine in the lower-level computer is used to acquire the fluorescent image, calculate the ratio value and store the images to the hard drive when commanded by RATTs. The raw image acquired from the Quantix camera is shown in Figure 3a. The image processing logic in the subroutine is:

- (1) The small square is extracted from the raw image around the laser trap position in both the left (red) channel and the right (green) channel as shown in Figure 3b and 3c respectively.
- (2) The two images are enhanced respectively by subtracting its background (the background intensity is assumed to be the summation of the maximum number of occurrence and the standard deviation found in the image histogram) as shown in Figure 3d for left channel and Figure 3e for the right channel.
- (3) The enhanced image of the right (green) channel is then converted to a binary image. The binary image serves as a mask on the images in Figure 3d and 3e.

The ratio value is the division of the left (red) channel over the right (green) channel after applying the mask in step 3. The average (6.7) is obtained as the ratio value of the fluorescent image of Figure 3a.

3. RESULTS

The add-on feature of fluorescent imaging in RATTs does not increase the RATTs processing time since the RATTs only changes a command variable when it requests an action from the lower-level system. The “track & trap” in RATTs can still keep up with the video rate of 30Hz when the tracked sperm is swimming inside the field of view, and only one image is lost when the sperm is relocated to the center via microscope stage movement.

One purpose of developing the add-on feature of fluorescent imaging in RATTs is to establish the baseline of sperm membrane potential. Under no laser interference, the sperm is tracked continuously and the ratio images are periodically captured. Figure 4a plots the ratio values of 10 randomly picked sperm, each tracked continuously for 29.3 seconds by RATTs. Fluorescent images, taken every 20 frames (0.667 seconds), are acquired just after a sperm is relocated via microscope stage movement to the user predefined location. Using the real-time fluorescent imaging processing and ratio calculation algorithm in Section 2.4, the average ratio values of those 10 sperm lie in the range of [7.5, 9]. Figure 4b shows the average ratio values in Figure 4a with respect to each sperm’s VCL. It is too early to draw any biological conclusions based on these preliminary results, but it is demonstrated that the add-on feature in RATTs can be used to study the baseline of sperm membrane potential.

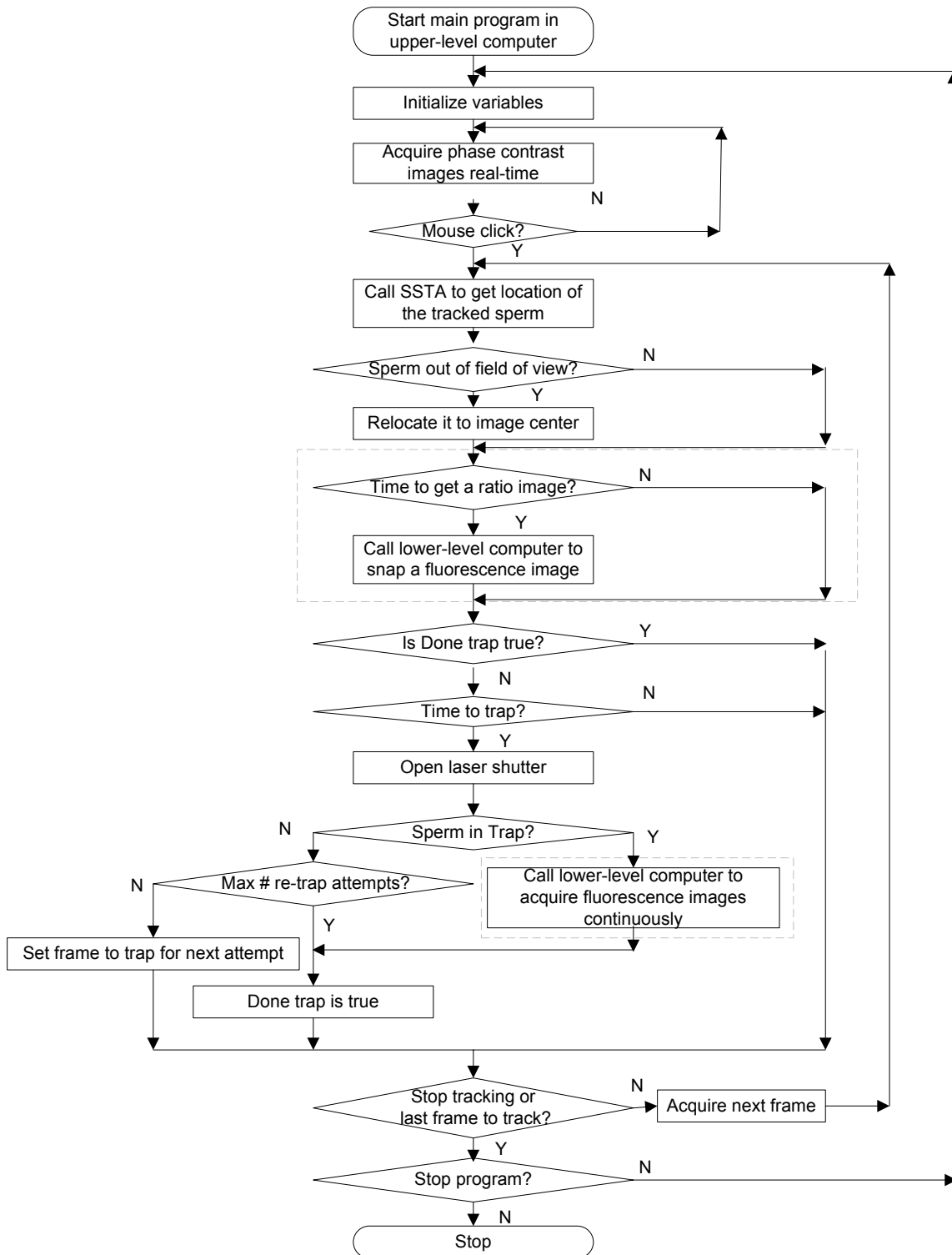


Figure 2 Flowchart of RATTs with add-on feature of fluorescent imaging

The add-on feature of fluorescent imaging in RATTs also can be used to quantify the effects of the optical barrier on the sperm energetics. The sperm of interest is normally tracked and trapped in the upper-level system as stated in Shi, et al. (2006). The fluorescent images of this sperm are acquired in the lower-level system using the logic shown in Figure 2. Figure 5 plots the ratio values of 4 different sperm with respect to pre-trap (time from 0 to 13.3 seconds), in-trap (time

from 13.3 seconds to 73.3 seconds), and post-trap (time from 76.02 seconds to 87.02 seconds). The sperm was held in the trap using 340 mW laser power in the focal volume. The ratio values appeared to decrease from pre-trap to in-trap to post-trap as shown in Figure 5 (a) (ratio values varied from 7.84 to 6.12 to 4.79) and (c) (ratio values varied from 6.29 to 6.24 to 5.30). The ratio value of sperm in Figure 5 (b) decreased 45% from pre-trap (6.85) to in-trap (3.06), but increased 23% from in-trap (3.06) to post-trap (3.76). The sperm in Figure 5 (d) escaped the laser trap at 56.56 seconds, and the ratio values appeared to increase from 6.07 (pre-trap) to 7.53 (in-trap). It is demonstrated that with the add-on feature of fluorescent imaging in RATTs, the effects of the optical barrier on the sperm energetics can be analyzed automatically and the motility parameters can also be obtained in the same experiment.

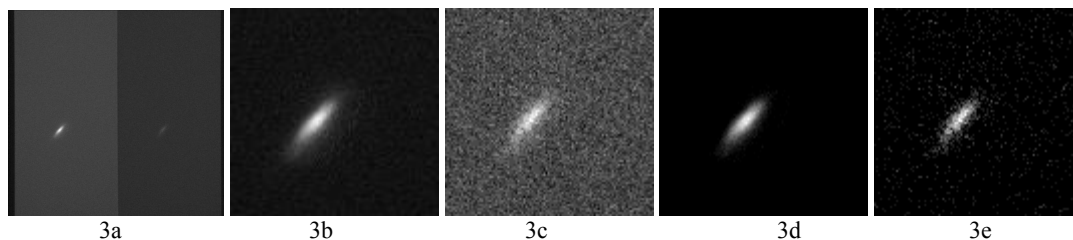


Figure 3 Image processing of fluorescent images (a) the raw image taken by the Quantix camera, (b) and (c) the enlarged partial image of the left and right channels respectively, (d) and (e) are the images after background subtraction.

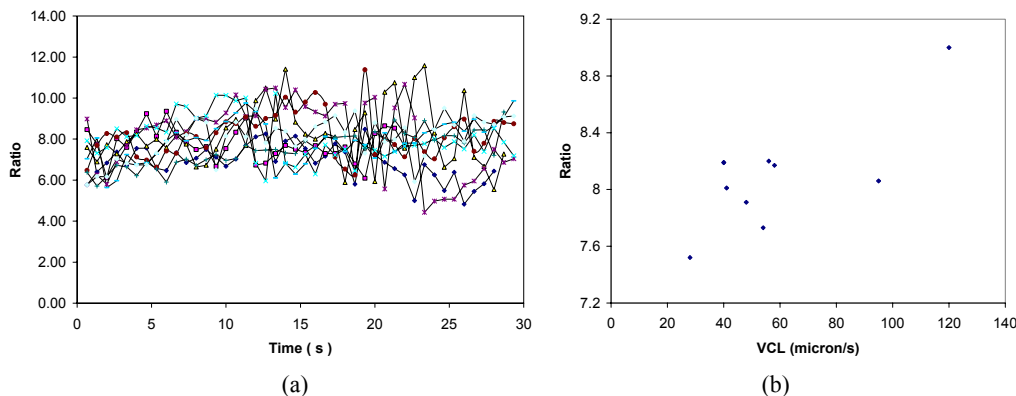
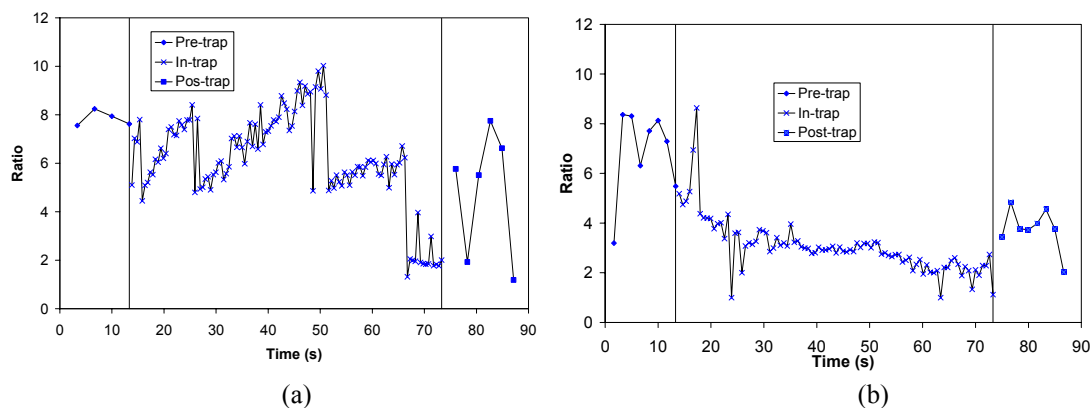


Figure 4 Results of 10 different sperm during continuous tracking. (a) ratio values of 10 sperm with respect to time, (b) ratio values of 10 sperm in (a) with respect to their VCLs.



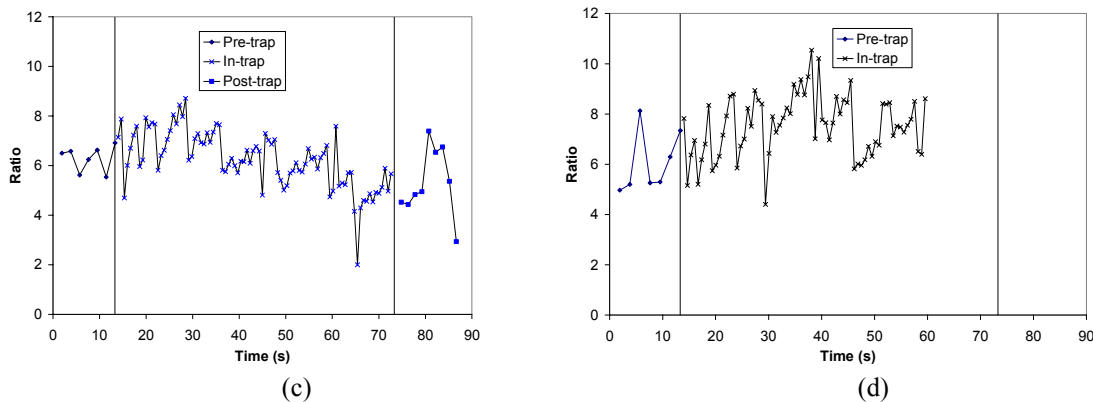


Figure 5 Ratio values of sperm in pre-trap, in-trap and post-trap durations. One fluorescent image is taken in every 1.33 seconds for the pre-trap and post-trap, and 0.67 for in-trap. (a) the average of ratio values are 7.84, 6.12, 4.79 for pre-trap, in-trap and post-trap respectively. (b) the average of ratio values are 6.85, 3.06, 3.76 for pre-trap, in-trap and post-trap respectively. (c) the average of ratio values are 6.29, 6.24, 5.30 for pre-trap, in-trap and post-trap respectively. (d) the average of ratio values are 6.07, 7.53 for pre-trap, in-trap respectively. It escaped the trap at 56.56 seconds.

4. DISCUSSION

The algorithm (RATTS with add-on feature of fluorescent imaging) and hardware integration presented in this study have achieved fast, automated measurement of the sperm mitochondria using a ratiometrically-encoded membrane potential fluorescent probe. The measurement of sperm swimming speed and the laser tweezers force are also obtained at the same time. A two-level computer system is constructed to do the three tasks at once: the upper-level system performs “track & trap”, and the lower-level system achieves fluorescent image acquisition, processing and storage. The two systems are connected through the crossover cable. The main program automatically runs the subroutine on the lower-level computer, and the subroutine opens up another one-way connection by polling the main program continuously. During the “track & trap”, the main program generates a command variable to “command” the lower-computer to respond when it is time to acquire a fluorescent image. The processing time in RATTS is not increased using this communication structure. Comparing the previous single-level system described in Mei, et al. (2005) the experimental throughput using the proposed two-level automatic system has been increased by an order of magnitude. Meanwhile, it was not possible to obtain the VCL in Mei, et al. (2005), since RATTS was not available, and the single-level computer was dedicated to the computationally intensive acquiring and storing of fluorescent images.

As can be seen in Figure 4, the fluorescent images of the sperm of interest can be acquired and the ratio values of the red channel over the green channel can be calculated in real-time when the sperm is tracked over a long period of time. The ratio values of 10 randomly picked sperm were quite consistent with respect to time (0-29.3 seconds) as shown in Figure 4 (a). The plot in Figure 4 (b) shows the relationship between the ratio values and the VCL. It is not possible to draw any biological conclusions based on these preliminary results. Additional tests need to be done in future experiments to observe the phenomena stated in Kasai et al. (2004) and Martinez-Pastor, et al. (2004). Meanwhile, the green channel signal needs improvement (to be much larger than the noise floor) which in future studies can be achieved by using different camera settings and even by binning to smaller size.

Figure 5 listed the ratio values of 4 different sperm before, during and after they were trapped by the laser. They were held by the constant laser power (340 mW in the focal volume) for 1 minute. The ratio values appeared to decline for sperm in Figure 5 (a) and (c), decreased from pre-trap to in-trap, but increased from in-trap to post-trap in Figure 5 (b), and increased from pre-trap to in-trap for the sperm in Figure 5d which was the sperm that escaped the laser trap by itself. Again, more experiments need to be conducted before any biological conclusions can be drawn. Compared with our previous work in Mei, et al. (2005), we now can obtain the ratio values of pre-trap and post-trap which makes it possible to gain a better understanding of the effect of the optical trap on the sperm energetics.

In summary the real-time single sperm tracking, laser trapping and radiometric fluorescent imaging system provides a platform to study the relationship between motility and energetics of sperm. This system can be used to test the sensitivity of fluorescent probes such as JC-1 and DiOC₂(3). The system can also be used to verify the probes ability to

respond to changes in the membrane potential when the cells are treated with agents that are known to affect mitochondria metabolism.

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