Automatic annular laser trapping: a system for high-throughput sperm analysis and sorting

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1. Introduction

Real-time computer-based tracking of live cells has become an important tool in biology. A robotic image-guided real-time respiratory tumor tracking system was discussed in Muacevic et al. 2007 [1]. The respiratory tracking error stated in [1] was less than 1 mm and the overall shape of the dose profile was not affected by target motion and/or phase shift between fiducial and optical marker motion. A real-time motion tracking algorithm was applied to ultrasonic strain imaging in Jiang & Hall, 2007 [2]. The modified predictive search block-matching algorithm in [2] can not only obtain high contrast strain images, but also reach 10 Hz frame rates. A real-time image-guided direct convective perfusion of intrinsic brainstem lesions was demonstrated by Lonser et al., 2007 [3]. It demonstrated that convection-enhanced delivery (CED) can be used to perfuse the brain and brainstem with therapeutic agents while simultaneously tracking their distribution using coinfusion of a surrogate magnetic resonance (MR) imaging tracer [3]. In the area of live cell tracking, sperm cells are one of the most difficult cell types due to their fast swimming speed (up to...
250 μm/s). The measurement of sperm motility has been a major focus of basic and clinical sperm research for over 25 years. The conventional assessment of human sperm, especially sperm movement, is a highly subjective assessment as stated in Nafisi et al., 2005 [4]. The tracking algorithm and a template matching method in [4] reduced error probability in finding and tracking sperm in various frames. Computer-assisted sperm analysis (CASA) systems have been commercially available for single sperm tracking since the mid-1980s. The goal of CASA systems has been to obtain objective data on sperm motility that can be used in research, human fertility clinics, and animal breeding programs. A detailed review of CASA can be found in Amann et al., 2004 [5] and Mortimer, 1994 [6]. In recent years, sex preselection by separating the male- and female-bearing spermatozoa using flow cytometry was used routinely in the agricultural industry [7, 8]. The optimized sorting conditions were discussed in Garner, 2005 [9] and Suh et al., 2005 [10]. Design and application of a microfluidic sperm sorter was also proposed for separation of motile sperm from non-motile ones [11–13]. Meanwhile, optical tweezers have been used as a tool to measure sperm swimming force and motility [14–19]. These studies determined that the minimal laser power needed to hold a sperm in the trap (threshold escape power) is directly proportional to the sperm’s swimming force. They also revealed a relationship between sperm motility and swimming pattern [19], and investigated the medical aspects of sperm activity [16, 17]. In our previous studies, a computer-based single sperm tracking algorithm was described [20] and subsequently a real-time single sperm tracking system was developed with an automated laser tweezers escape power assay [21]. To dynamically monitor changes in sperm swimming behavior under the influence of a laser beam, a continuous 3-D ring-shaped trap was developed [22]. In order to increase the accuracy and efficiency of analysis and sorting of sperm, a real-time automated sperm tracking and annular trapping system is described in this paper.

2. Material and Methods

2.1. Sperm preparation

Semen samples were collected from two primates: human (Homo sapiens, four males, pooled frozen samples); and western lowland gorilla (Gorilla, 1 male, 1 frozen sample) and prepared as described previously [23]. Briefly, human semen samples were frozen according to published protocols [24–26] and prepared for analysis using a twice-wash protocol [27, 28]. Gorilla semen was frozen according to a published protocol [29] and thawed for 8 s in 50 °C water bath before analysis. Gorilla semen was suspended in BWW + BSA (1 mg of BSA per 1 mL of BWW) for analysis. Human sperm were suspended in modified Human Tubal Fluid (mHTF), HEPES buffered (osmolarity 272–288 m Osm kg⁻¹ water, pH of 7.3–7.5), with 5% serum substitute supplement (SSS) and filtered through 0.2 mm syringe filter (Irvine Scientific, Santa Ana, CA). Sperm samples of 30,000 sperm ml⁻¹ of media dilutions were loaded into a 120 μm-thick chamber with a glass slide as the top and a No. 1 cover-slip as the bottom. The sample was mounted into a microscope stage holder and kept at room temperature during the experiment.

2.2. Hardware system

The annular laser trapping optical system [22] is shown in Figure 1. Briefly, a CW Ytterbium fiber laser with 1070 nm wavelength (PYL-20M, IPG Photonics, Oxford, MA) is used as the laser source. An axicon-lens pair (axicon1-FL) is used to generate an annular focus that is conjugate to the ring-shaped trap at the specimen plane. After the tube lens (TL), the laser beam is sent into an inverted microscope (Axiovert 200 M, Zeiss, Germany), and directed to the objective (63 × oil immersion, NA = 1.4, Zeiss, Germany) by the dichroic mirror. The ring-shaped trap obtained at the specimen plane has a diameter of 100 μm, which could accommodate up to 70 human sperm, whose head diameter is approximately 5 μm.

Compared to the manually controlled hardware trapping system [22], the following modifications were made to achieve real-time automatic annular sperm trapping: (1) The fiber laser was connected to the computer through a serial cable and the serial commands provided by the laser manufacturer are used to control the laser power from the main controller (Intel Core 2 CPU, 6700 @ 266 GHz, Alienware Corp, Miami, FL); (2) Specimens were mounted in a motorized X-Y stepper stage (Ludl Electronic Products Hawthorne, NY) controlled with a PXI-7344 motion controller (National Instruments, Austin, TX) and a MID-7604 power drive (National Instruments); (3) A mechanical shutter (Uniblitz LS6ZM2, Vincent Associates, Rochester, NY) was added in the laser path and controlled by a shutter controller (Uniblitz VMM-D3, Vincent Associates), and the shutter controller was connected to an analog channel of the motion controller PXI-7344; (4) A Hamamatsu CCD was connected to the computer through an image acquisition board (IMAQ PXI-1409, National Instruments); both the motion controller and IMAQ board were mounted in a PXI chassis (National Instruments), which was connected to the host computer through two MXI4 boards (one in the PXI chassis, the other in the host compu-
ter) through the MXI-3 fiber-optic cable (National Instruments). The block diagram of the hardware system is shown in Figure 2.

2.3. Software Design

Based on the hardware modification, the phase contrast images of swimming sperm (as shown in Figure 3) are digitized to the computer at video rates. The custom algorithm written in the Labview 8.2 (National Instruments) language creates a region of interest (ROI, green square box as shown in Figure 3) centered about a sperm in response to a mouse click. The annular trap is drawn on every new captured image (yellow circle, as shown in Figure 3). The major image processing steps, such as image contrast enhancement, image segmentation, collision detection, post-collision analysis, were discussed previously [20]. The microscope stage is controlled in real-time to keep the sperm of interest in the field of view. For the single laser trap [21, 23], it was relatively easy to relocate the sperm of interest to the laser point by moving the microscope stage. With two specific different entering angles, the annular trap can serve two purposes: (1) as a normal entering angle, the annular trap can function as a “speed bump” (for general sorting purpose); (2) as a tangential entering angle, the annular trap can

Figure 1 (online colour at: www.biophotonics-journal.org) Optical setup for annular trap.

Figure 2 Real-time automated sperm tracking and annular trapping system.
change the sperm swimming behavior (some sperm may start swimming along the annular trap). The steps for relocating a sperm to observe its response to the trap are:

1. Move the sperm of interest outside the annular trap.
2. Turn on the laser by opening the mechanical shutter in the laser path.
3. Cause the sperm of interest to encounter the annular trap with either a normal angle (0 degrees) or a tangential angle (90 degrees) with respect to the trap.

To achieve these three goals, the assumption is made that the sperm does not make a sudden or significant change in swimming direction. For the two species used, human and gorilla, the sperm swim in a relatively smooth trajectory, such as the human sperm shown in Figure 4(a). Note that a short time (for instance, 90 ms) is needed to move the microscope stage when relocating the sperm near the annular trap. It is also better to allow the sperm to swim toward the annular trap after the trap is turned on (by opening the mechanical shutter). The following parameters were defined to move the sperm of interest to a point near the annular trap with a preferred angle:

1. Current frame: \( i \) (\( i = 100 \), while 100 is set as the 1st frame to start relocation process)
2. Current sperm position and current velocity: \((x_i, y_i)\) and \(V_{CL_i}\)
3. Position 20 frames before: \((x_{i-20}, y_{i-20})\)
4. Center and radian of the annular trap \((x_C, y_C)\) and \(R\) respectively.
5. Next frame: \( i + 1 \). It is approximately 90 ms after the current frame \( i \).
6. Projecting time for the sperm swimming from the point near the annular trap to the annular trap: \(T_{p}\).

The logic to relocate the sperm with a tangential entering angle (the logic for a normal entering angle is similar):

1. Calculate the sperm swimming angle \( \alpha \) as shown in Figure 4(b).

\[
\tan \alpha = \frac{y_i - y_{i-20}}{x_i - x_{i-20}}; \\
\sin \alpha = \frac{y_i - y_{i-20}}{\sqrt{(x_i - x_{i-20})^2 + (y_i - y_{i-20})^2}}; \\
\cos \alpha = \frac{x_i - x_{i-20}}{\sqrt{(x_i - x_{i-20})^2 + (y_i - y_{i-20})^2}}.
\]
(2) Draw a line tangential to the annular trap and parallel to the swimming angle. There will be two lines available, and only one is chosen as shown in Figure 4(b). Define the point of tangency as \((x_{i+1}, y_{i+1})\). That is the ideal point the sperm should be relocated to. The coordinate of \((x_{i+1}, y_{i+1})\) is:

\[
xx_{i+1} = x_c - R \sin \alpha; \quad yy_{i+1} = y_c + R \cos \alpha.
\]

(3) Note that it takes 80 ms to relocate the sperm (by moving the microscope stage) from point \((x_i, y_i)\) to point \(xx_{i+1}, yy_{i+1}\) and 10 ms to turn on the annular trap. The sperm may be relocated to the position inside of the annular trap if this 90 ms time delay is not considered in the algorithm. Therefore, a distance for the sperm to swim toward the trap in this time delay is projected assuming that the sperm will still be swimming at the same angle \(\alpha\). The algorithm presented in this paper requires the user to set the projecting time \(T_d\) large enough (usually larger than 90 ms) for the sperm to swim toward the annular trap.

(4) Define the new coordinate consider the projecting trajectory as \((x_{i+1}, y_{i+1})\)

\[
\begin{align*}
xx_{i+1} &= xx_{i+1} - T_d \times VCL_d \times \cos \alpha; \\
yy_{i+1} &= yy_{i+1} - T_d \times VCL_d \times \sin \alpha.
\end{align*}
\]

The logic works well for sperm that swim with a relative large forward movement. For circular spinning sperm and slow swimming sperm with large lateral head displacement, the swimming angle will be misleading when using the equation in Step (1). Instead, the crossing point of the line connecting \((x_c, y_c)\) and \((x_{i-20}, y_{i-20})\) with the annular trap is assumed to be \((x_{i-20}, y_{i-20})\):

\[
\begin{align*}
x_{i-20} &= x_c - R \frac{x_{i} - x_c}{\sqrt{(x_{i} - x_c)^2 + (y_{i} - y_c)^2}}; \\
y_{i-20} &= y_c - R \frac{y_{i} - y_c}{\sqrt{(x_{i} - x_c)^2 + (y_{i} - y_c)^2}}.
\end{align*}
\]

Then Step (2) and (3) are followed to calculate \((x_{i+1}, y_{i+1})\).

3. Results

The system described here has been successfully applied to analyzing and sorting sperm of two vertebrate species: human and gorilla. There are five possible responses sperm could have after encountering the annular trap: stop, slow down, no detectable change, speed up, slide and swim along the annular trap (later two responses refer to sperm changing swimming trajectory to an arc of the annular trap).

In this section, three examples are given for the last three types of human sperm response. The sorting results of human and gorilla sperm are compared at the end of this section.

1) Sperm speeding up after encountering the annular trap.

Some sperm will increase in speed after encountering the annular trap. Several video images (saved real-time to the hard-drive) of a randomly picked sperm are presented in Figure 5. The user clicked the sperm on the image screen and a region of interest (ROI) box was drawn as shown in Figure 5(a). After 3.3 seconds (100th frame), sperm tracking has begun (as shown in Figure 5(b)); the algorithm is designed to relocate the sperm near the annular trap with a 0 degree angle (normal) entering the trap. Next, the laser is turned on (as shown in Figure 5(c)). The laser power was 12.1 W, the read-out from the laser controller (displayed on the image in Figure 5(c)), and 18mW per sperm at the sample plane. The sperm did not stop, slow down, nor change its swimming direction as shown in Figure 5(d). It did speed up with respect to the previous swimming direction to the position shown in Figure 5(e) at 6.6 seconds (200th frame). At that time, the laser power had been turned off (after the sperm passed through the annular trap). The algorithm then underwent an attempt to trap the sperm by relocating the sperm two more times before the user stopped the “track and trap” of that sperm. VCL is defined as the curvilinear velocity from frame to frame. The average value of the pre-trap VCL (from the 1st frame to the 100th frame) is 50 \(\mu\)m/s. The average VCLs of each attempt are 64, 62 and 76 \(\mu\)m/s as shown in Figure 5(f). For this sperm, the annular trap increased its velocity by 20–50%.

2) Sperm sliding along annular trap (changing its swimming trajectory to an arc for a short duration).

Some sperm will experience sliding along the annular trap for a very short duration before swimming away from it. The behavior of sperm can be analyzed using the recorded laser power and the sperm’s position. Several images of a randomly picked sperm sliding along the annular trap are presented in Figure 6. The 1st captured image as a result of the user mouse click is shown in Figure 6(a). The sperm was swimming in upper-left direction. The image before the 1st attempt to relocate the sperm to the annular trap (after 3.3 seconds) is given in Figure 6(b). An image after relocating the sperm at the 1st attempt is shown in Figure 6(c). Note that the entering angle was at a tangential angle instead of a normal angle to the annular trap (set by the algorithm). Sperm slid along the trap for 21 frames (0.7 seconds) and swim toward the upper-left corner as shown in the image 1.5 seconds after the 1st attempt (Figure 6(d)). An image of relocating the sperm to the annular trap at
the 2nd attempt is shown in Figure 6(e). The sperm slid along the trap for 72 frames (2.4 seconds) after the 2nd attempt. The image before the sperm swam away from the annular trap is shown in Figure 6(f). The sperm swam toward the left afterwards. The image two seconds afterwards is shown in Figure 6(g). The sperm was relocated to the annular trap at the 3rd attempt as shown in Figure 6(h). The sperm slid along the annular trap for 159 frames (5.3 seconds). The image before the sperm swam away is shown in Figure 6(i). The average values of the pre-trap VCL, 3 VCLs for sliding along the trap and 2 VCLs in between are 47, 60, 60, 52, 57 and 60 m/s respectively as shown in Figure 5(j).

3) Sperm swimming along annular trap with laser power decay.

In this experiment, some sperm swim along the annular trap for a relatively long time, such as 20 seconds, experiencing the case of fatigue [22], and some swim out of the annular trap if the laser power is reduced to a lower value, such as the power gradient case [22]. In order to precisely calculate at what laser power the swimming-along sperm escape the annular trap, the algorithm steps down the laser power automatically (1 W at the laser controller read-out which equals 1.5 mW per sperm on the annular trap) at the user pre-defined time interval (set as 100 frames in the algorithm). The precise laser power of each image frame is read from the laser controller to the computer and is saved to the video-rate data file. Three randomly selected cases are displayed in Figure 7. The sperm in Figure 7(a) swam at 35 m/s before it was relocated to the annular trap, and slowed down to 32 m/s and 25 m/s when the trapping power was reduced from 24 mW to 22.5 mW per sperm. Then the sperm sped up to 32 m/s at 21 mW, slowed down again for powers 19.5 mW and 18 mW, but gradually increased in speed again until it swam out from the annular trap at 9 mW per sperm. The post-trapping VCL of 34.68 m/s was equivalent to the pre-trap VCL value. This serves as a good indication of the non-invasive feature of the laser trap. A
Figure 6 (online colour at: www.biophotonics-journal.org)
Sperm sliding along the trap: (a) The 1st captured image as a result of the mouse click. (b) Image before the 1st attempt to relocate sperm to the annular trap (100th frame). (c) Image after the 1st attempt to relocate the sperm to the annular trap with a tangential entering angle. (d) Image of sperm 1.5 seconds after the 1st attempt. (e) Image at the 2nd attempt of relocating sperm to the annular trap. (f) Image of the sperm sliding along the trap for 72 frames after the 2nd attempt and before swimming away. (g) Image of the sperm 2 seconds after swimming away from the trap. (h) Image at the 3rd attempt to relocate sperm to the annular trap. (i) Image of the sperm sliding along the trap at the 159th image after the 3rd attempt. (j) VCLs of the sperm pre-trap, sliding, and post each attempt.
similar speed pattern was observed in the sperm shown in Figure 7(b) except that this sperm did not escape from the annular trap before the laser power was reduced to zero. Following the reduction of the laser power to zero, the swimming speed returned to the pre-trap value. In the power decay experiment, some sperm exhibit VCL changes after their swimming trajectories were restricted by the annular trap. These sperm could not escape from the annular trap until the trap power was reduced to zero as shown in Figure 7(c). The post-trap VCL of the sperm was 40% of the pre-trap VCL in Figure 7(c).

4) Sorting of human and gorilla sperm.

The sorting of human sperm using a manual system has been discussed in a previous study [22]. In this paper, we described an automatic system to achieve this goal. The three most commonly used parameters are discussed in this section: (1) curvilinear velocity (VCL) in $\mu$m/s, (2) smooth path velocity (VAP) in $\mu$m/s, (3) amplitude of lateral head displacement (ALH) in $\mu$m.

**Figure 8** (online colour at: www.biophotonics-journal.org) Parallel human sperm sorting using automatic annular laser trapping system with 21.4 mW per sperm. The error bars of VCL, VAP and ALH corresponds to $\pm1$ standard deviation. The sample size is 183.

**Figure 9** (online colour at: www.biophotonics-journal.org) Parallel gorilla sperm sorting using automatic annular laser trapping system with 21.4 mW per sperm. The error bars of VCL, VAP and ALH corresponds to $\pm1$ standard deviation. The sample size is 81.
In this study, an automatic annular laser trapping system has been used to separate human and gorilla sperm, respectively, with different motilities based on these three parameters. Two groups of sperm are classified: The “fast” group is defined as sperm that swim through the annular trap with no detectable change in velocity (both VCL and VAP). The “slow” group represents sperm whose swimming pattern is considerably affected by the annular trap, including those that: (a) swim along the annular trap, (b) slide along the annular trap for a short time and swim away, (c) stop by the annular trap (not included in the data analysis). Human sperm (pooled from different males) with an average 21.4 mW per sperm were analyzed (Figure 8). The data sets are compared using Matlab; a Lilliefors test is first performed to check for normal distribution (at the 5% significant level); to compare different data sets, the t-test is used if both data sets are normal, or the Wilcoxon rank-sum test otherwise. The VCL, VAP, and ALH values in the “slow” group are statistically different from those in the “fast” group ($p$-value of 8.9 x $10^{-13}$ for VCL, 1.98 x $10^{-14}$ for VAP, and 9.08 x $10^{-14}$ for ALH). Figure 9 shows sorting of gorilla sperm (same day experiment from same vial) under average 21.4 mW per sperm. A gorilla sperm has similar geometrical shape to a human sperm. With large sample size, the average velocities of gorilla sperm were less than those of human sperm as shown in [23]. Due to the small sample size here in this paper, we don’t see the difference in velocity between those two species. The same statistical analysis is applied to gorilla sperm data. Just like human sperm data, the VCL, VAP, and ALH values of gorilla sperm in the “slow” group are statistically different from those in the “fast” group ($p$-value of 5.2 x $10^{-10}$ for VCL, 1.48 x $10^{-7}$ for VAP, and 3.81 x $10^{-6}$ for ALH). As we see in Figures 8 and 9, the average values of VCL, VAP, and ALH for the two species are very close for the same laser power (21.4 mW). Table 1 lists the number of sperm in each group (fast vs. slow) with different incidence (normal vs. tangential). The slow group is also divided into two sub-groups: sliding along the annular trap and swim along the annular trap.

### Table 1 List of sample size in different categories (Human vs. Gorilla, Slow vs. Fast, Normal vs. Tangential Incidence).

<table>
<thead>
<tr>
<th>Species</th>
<th>Group Name</th>
<th>Swim Pattern</th>
<th>Number of Normal Entering Angle</th>
<th>Number of Tangential Entering Angle</th>
<th>Total Sperm Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Slow</td>
<td>Sliding with annular trap</td>
<td>3</td>
<td>43</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Swim along annular trap</td>
<td>6</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>Fast</td>
<td>17</td>
<td>71</td>
<td>88</td>
</tr>
<tr>
<td>Gorilla</td>
<td>Slow</td>
<td>Sliding with annular trap</td>
<td>1</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Swim along annular trap</td>
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<td>32</td>
<td></td>
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<tr>
<td></td>
<td>Fast</td>
<td>Fast</td>
<td>2</td>
<td>36</td>
<td>38</td>
</tr>
</tbody>
</table>

### 4. Discussion and future work

The annular laser trap system described here can perform fast, accurate sperm analysis and sorting. Instead of using a joystick to manually locate the sperm to the outside of the annular trap and manually turn on the laser, the system we describe can not only perform those two steps automatically but also can relocate the sperm with two specified entering angles: (1) normal (the trap may function as a speed bump) and (2) tangential (the trap may change the sperm behavior). The data file saved in real-time contains the information of sperm coordinates, velocity value, laser status (on/off), and laser power value for the current image frame. For the cases when the sperm slides along the annular trap longer than the user-predefined duration, the laser power is stepped down automatically at the user pre-defined time interval so that the precise laser power of each image frame is read out from the laser controller to the computer and is saved to the data file at video rates. The average velocity can be calculated accurately for each laser level. There is no post-experiment image processing needed and the total data analysis is reduced at least by an order of magnitude compared to our previous set-up [22].

Three major responses of sperm when encountering the annular trap have been described in detail in this paper: speeding up, sliding, and swimming along the annular trap with longer durations. First, in the case of speeding up, the sperm velocity passing the annular trap can be compared with that of the pre-trap velocity using the saved data infor-
mation. This system can also be setup to relocate the sperm to the annular trap multiple times in order to observe if the same behavior repeats as shown in Figure 5. Second, in the case of sliding, the system is able to identify the exact frames when the sperm changes its swimming trajectory to an arc of the annular trap and also accurately calculate the velocity during that duration. Last, in the case of swimming along the annular trap with longer duration, the laser power is stepped down automatically to determine the sperm escape power and observe the velocity variations of sperm swimming along the annular trap.

An important application of the annular trap system is for sperm sorting. This paper compared three motility parameters of data shown in human sperm (Figure 8) and gorilla sperm (Figure 9): VCL, VAP, and ALH. Two groups are classified based on the sperm response to the annular trap: The “fast” group is defined as sperm that swim through the annular trap with no detectable change (both VCL and VAP). The “slow” group represents sperm whose swimming pattern is considerably affected by the annular trap, including those that are slowed down, stopped, or which swim along the annular trap.

For future work, a chemo-attractant, such as ovary extracts, can be placed to the center of the annular trap. In literature, chemotaxis is considered a critical feature of sperm in response to the diffusion gradient of chemicals released by the egg and surrounding cells of the cumulus oophorus. Sperm should start approaching the chemoattractant from all directions. As a result, the annular trap system could be ideal to sort sperm according to their chemotactic response.

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