A Noninvasive, Motility Independent, Sperm Sorting Method and Technology to Identify and Retrieve Individual Viable Nonmotile Sperm for Intracytoplasmic Sperm Injection

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Abbreviations and Acronyms

DEP = dielectrophoresis

ICSI = intracytoplasmic sperm injection

OET = optoelectronic tweezers

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For another article on a related topic see page 2586.

Purpose: For intracytoplasmic sperm injection in the absence of sperm motility it can be virtually impossible to distinguish viable from nonviable sperm. A reliable means to identify viable nonmotile sperm is needed and would likely improve the intracytoplasmic sperm injection success rate. Optoelectronic tweezers are a new technology that uses light induced dielectrophoresis fields to distinguish individual live cells from dead cells. We assessed the ability of optoelectronic tweezers to distinguish viable from nonviable individual nonmotile human sperm.

Materials and Methods: Fresh semen specimens from 6 healthy men were suspended in an isotonic sucrose/dextrose solution and incubated with 0.4% trypan blue dye (Sigma-Aldrich®). Within 15 minutes we randomly selected 5 motile and 50 nonmotile sperm, including 25 trypan negative, followed by 25 trypan positive sperm, under 200× magnification for optoelectronic tweezers assay. We recorded the individual sperm response (attraction or repulsion) to the optoelectronic tweezer field and trypan staining status.

Results: From each subject 55 unwashed sperm were individually assayed for a total of 330. All motile sperm were attracted to optoelectronic tweezers. Of 150 trypan negative (viable) sperm 132 (88%) were attracted to the optoelectronic tweezer field with 0.88 sensitivity (95% CI 0.82–0.93) vs that of the trypan blue assay. All 150 trypan positive (nonviable) sperm were repulsed by or neutral to the optoelectronic tweezer field with 1.0 specificity (95% CI 0.98–1.00) vs that of the trypan blue assay. Type I error equaled 0 and overall assay agreement was 94%.

Conclusions: The optoelectronic tweezer assay can distinguish viable from nonviable nonmotile viable sperm with sensitivity comparable to that of the trypan blue assay and equal specificity. Optoelectronic tweezers are a promising means of selecting sperm for intracytoplasmic sperm injection.

Key Words: testis; spermatozoa; sperm injections, intracytoplasmic; electrophoresis; optical devices

DURING natural conception and in vitro fertilization natural selection determines which motile sperm successfully fertilizes the oocyte. However, this mechanism of natural selection is bypassed by ICSI since a single sperm is manually injected directly into the oocyte. Thus, any sperm regardless of quality or viability can potentially deliver its genome to the oo-

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cyte and resulting embryo. Hence, it is essential that only viable and ideally only the healthiest sperm be selected for ICSI. Inadvertent use of dead or dying sperm likely contributes to unfavorable ICSI outcomes, such as immediate or delayed embryo failure, and genetic mutations and/or epigenetic effects in the developing embryo,[1–4] potentially resulting in an increased incidence of birth defects.[2,4–8]

Sperm motility and morphology are currently the most commonly used criteria for in vitro sperm selection. However, sperm motility is not a robust predictor of sperm quality and it does not exclude significant DNA damage[1,5,9] or waning viability. Sperm morphology is also a poor index of sperm viability.[3]

When sperm motility is limited or absent, sperm selection for ICSI is challenging. Whether a particular sperm is viable can be a matter of chance. Also, when overall motility is decreased in a sample, individual sperm motility is less predictive of quality since poor overall motility in infertile men is associated with an increased mean index of DNA damage and a lower live birth rate after ICSI.[6,7,10]

The hypo-osmotic swelling test was developed to assess the viability of nonmotile sperm for ICSI.[11] However, its interpretation is subjective. It is not often used due to limited sensitivity and specificity, particularly in cryopreserved sperm.[12] Studies suggest that sperm swelling may induce oxidative stress, causing sperm DNA damage and structural damage.[13–15] Numerous chemical assays are available to assess sperm viability and quality (trypan blue and eosin Y vital dye exclusion tests, and fluorescent in situ hybridization), cell DNA damage (Comet assay), DNA integrity (sperm chromatin structure) and apoptosis (TUNEL assay). However, each test is invasive and toxic to the cell, limiting use to diagnostic applications and precluding use in sperm reserved for ICSI.[6,7]

Human reproduction and ICSI operate at the level of single gametes, that is only 1 sperm joins 1 oocyte. It follows that the limit of resolution for technologies to guide sperm selection should be the individual sperm. However, except for the hypo-osmotic swelling test all current sperm sorting techniques operate in sperm samples (not the same sperm for ICSI) or in groups of sperm (not individual sperm). This decreases their net predictive value. In light of these limitations a noninvasive means is needed to determine whether a single individual sperm cell is viable at ICSI.

Dielectrophoresis refers to the motion of neutral particles or cells in response to external nonuniform alternating current electric fields. OET is a novel experimental means to produce DEP fields using only projected light patterns instead of the traditional approach using a fixed electrode. DEP and OET distinguish live from dead cells (other than sperm) based on the different intrinsic dielectric properties of the latter.[16–19]

The key innovation of OET is that it is optically based. A DEP field is generated by patterned light projected on the OET device containing cells. The light pattern controls an alternating electric field, which produces the OET-DEP field. Inside the OET device cells are suspended in a solution with conductivity significantly less than that of a viable cell. Since viable cells have an electric gradient, they are polarized by the OET field patterned on the suspension and attracted toward the OET field.[16,20] Because dying or dead cells lose the electric gradient, they become at most only weakly polarized by the OET field. At the 10^5 Hz operating frequency of our device this results in a weakly repulsive or neutral response to the OET field (fig. 1).

OET provides several advantages over fixed electrode generated DEP. The DEP field produced can assume any size, shape, pattern or voltage desired[16] and can be moved in real time to the site of any cell in the sample. These features render the OET assay versatile and simple to perform. Cells can be assayed individually or together for higher throughput.

We hypothesized that OET could be used to distinguish viable nonmotile sperm from nonviable nonmotile sperm in an unwashed semen sample. A working prototype micro-fluidic sperm sorting platform was manufactured for this pilot study. To our knowledge this is the first reported application of OET to manipulate and assay sperm.

MATERIALS AND METHODS

OET apparatus components and assembly have been previously described.[16] Sperm sorting is performed in a micro-fluidic chamber bounded by 2 specialized glass slides (fig. 2, *A*). The lower slide is formed by a 2.5 × 1.5 cm × 1 mm glass slide with an inner surface coated by a 200 nm film of indium-tin-oxide and a 1 μm film of amorphous silicon (fig. 2, *B*). The upper slide is also formed by a glass slide with an inner surface coated by indium-tin-oxide film. The 2 surfaces of the OET micro-fluidic chamber are separated by a 100 μm spacer, creating a chamber space into which the suspension of sperm cells to be assayed is delivered.

The OET sperm sorting chip was placed on an XYZ micro manipulator connected to a mechanical stage drive, which allowed the stage to be moved manually, under a custom-built 5× objective upright DIC microscope connected to an XCD-X710CR closed circuit digital camera (Sony®) (fig. 3). Fresh ejaculate specimens produced by masturbation after 2 to 3 days of abstinence were obtained from 6 healthy male volunteers. All samples were maintained at room temperature and assayed within 6 hours of production. Specimen adequacy was confirmed by motile sperm under microscopy. The viability of all sperm was determined by simultaneous trypan blue vital dye exclu-

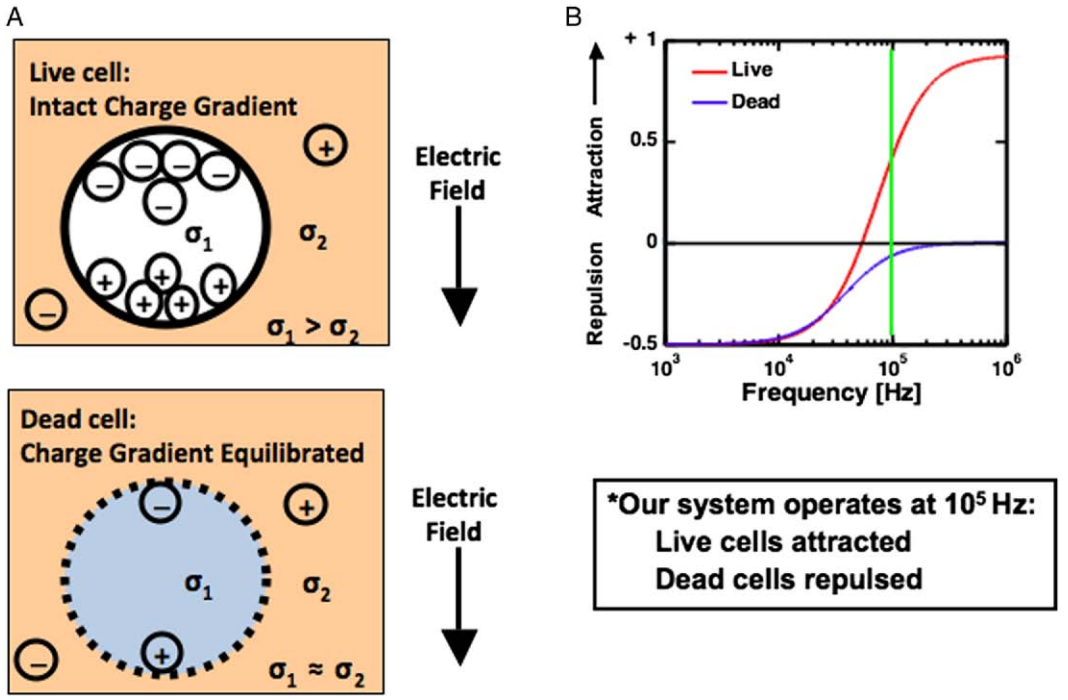

Figure 1. *A*, live cells are highly polarized in electric field, which induces cell dipole moment formed by ions in cell. Ion concentration gives cell conductivity (σ) greater than that of surrounding medium. Dead cells do not maintain ionic gradient across membranes and organelles. Thus, they do not have such strong dipole moment. *B*, normalized OET response curve. Live cells can experience attractive (positive) forces toward high electric field region. Dead or dying cells have weakly or no repulsive (negative) forces.

sion assay. With the trypan assay viable sperm exclude the dye and remain unstained while dead sperm allow passive dye uptake and become blue. An aliquot of each sample was mixed in a 1:1 volume ratio with 0.4% trypan blue dye in deionized water and incubated at room temperature for 3 minutes. The semen/trypan mixture was diluted approximately 100× by adding an isotonic, minimally conductive solution consisting of 8.5% sucrose and 0.3% dextrose in deionized water. The conductivity of the diluted solution containing sperm was adjusted to 6.5 mS/m in all samples.

A 20 μl aliquot of each trypan exposed semen sample was delivered into the micro-fluidic chamber of the ultraviolet sterilized OET sperm sorting chip. Within 10 minutes of incubation with trypan dye we assayed 55 individual sperm per each of the 6 donor samples. Five randomly selected motile sperm per sample served as positive assay controls. If each could be manipulated by the OET field, the OET device was confirmed to be working. Thereafter we assayed only grossly intact single and not clumped nonmotile sperm, which differed from each other only by positive or negative trypan staining status, from each

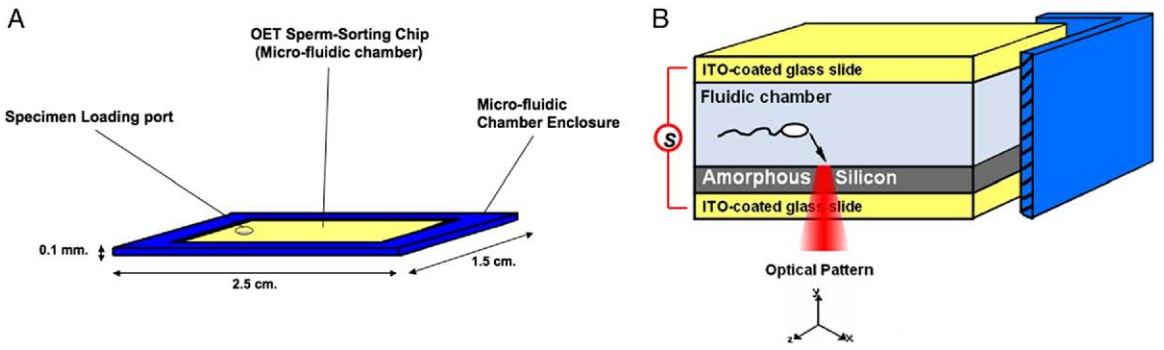

Figure 2. *A*, sperm sorting chip consists of glass slide containing micro-fluidic chamber filled with solution containing cells to be sorted. Prototype does not have outlet ports to retrieve sorted cells but this could be incorporated. *B*, micro-fluidic chamber in sperm sorting chip consists of lower 2.5 × 1.5 × 1 mm glass slide coated with 200 nm indium-tin-oxide (*ITO*) and 1 μm amorphous silicon films. OET upper surface is also glass slide with indium-tin-oxide film. Two OET surfaces are separated by 100 μm spacer creating fluidic chamber for sperm manipulation.

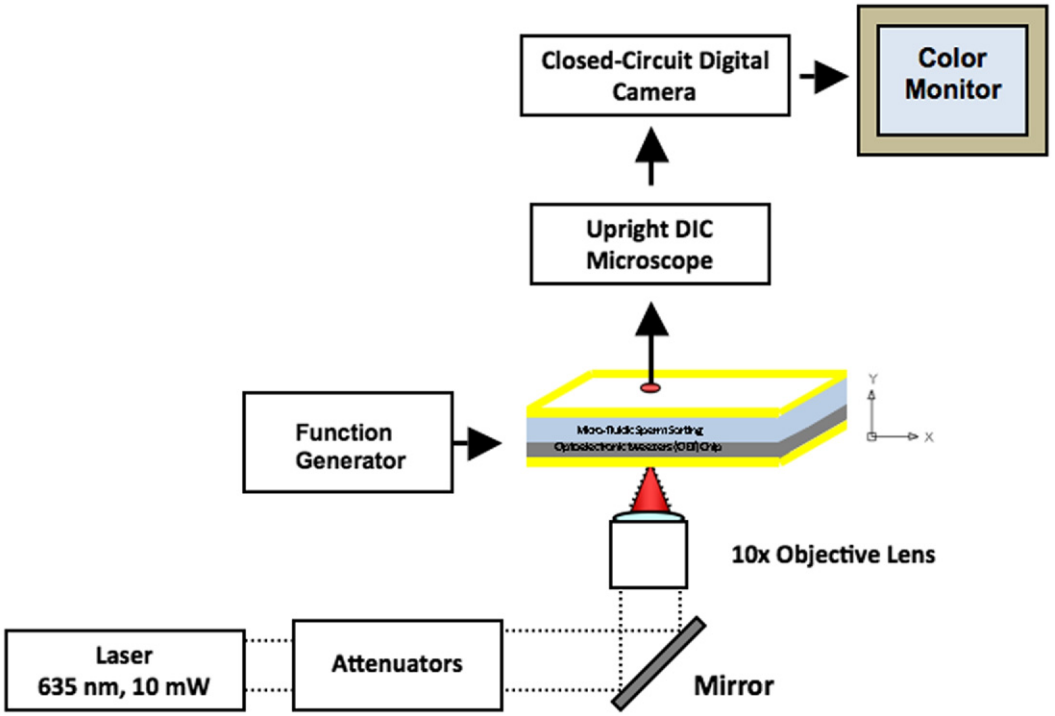

Figure 3. Primary OET sperm sorting platform components include micro-fluidic sperm sorting chamber in slide chip (*Function Generator*) mounted on movable stage of differential interference contrast microscope. Laser energy/light is projected on sperm sorting micro-chamber (chip) from beneath stage. Digital camera connected to microscope captures image and displays it on color monitor.

sample, including 25 nonmotile trypan negative sperm, followed by 25 nonmotile trypan positive sperm. We recorded the response of each sperm to the OET field with attraction indicating a positive and repulsion indicating a negative response. The OET assay was repeated 3 times per sperm and results were averaged. After the OET assay we evaluated sperm morphology and motility to assess evidence of damage. Study design and methods were reviewed and approved by our institutional committee on human research.

Trypan blue served as the gold standard reference assay while OET was classified as the experimental assay. Sensitivity, specificity and overall assay agreement were calculated with the exact 95% CI.

RESULTS

All sperm had the same gross appearance/morphology before and up to 15 minutes after the OET assay. No visible damage was detected.

Motile sperm were visualized in all samples. A total of 330 individual sperm were individually assayed (fig. 4). All motile sperm (5 per sample) were trypan blue negative (viable), responded positively to OET and remained motile after assay, resulting in total agreement with trypan blue assay and indicating 100% sensitivity and specificity (95% CI 0.88–1.0) vs trypan blue (fig. 4, *A*).

Of 150 trypan negative nonmotile sperm 132 (88%) were classified as alive by OET due to a positive response to the OET field. Viable nonmotile sperm migrated spontaneously to the center of the OET field, where OET field strength is most concentrated, while dead nonmotile sperm were weakly repulsed by the OET field (fig. 5, *A*). Due to attraction to the field each OET positive sperm could be moved (dragged) about the sorting area in real time simply by moving the OET field site. The trypan assay served as the reference standard. On OET assay 18 trypan negative sperm (12%) were classified as dead (type II error). Mean OET assay sensitivity was 88% (95% CI 0.82–0.93). All 150 trypan positive nonmotile sperm responded negatively to OET and were mutually classified as dead. Mean specificity was 100% (95% CI 0.98–1.0) and type I error equaled zero. Mean interassay agreement for nonmotile sperm was 94% (range 84% to 100%) (fig. 4, *B*).

DISCUSSION

The results of this pilot study suggest that the proposed OET based sperm assay platform can identify individual viable nonmotile sperm in situ from among otherwise indistinguishable nonviable sperm in a fresh, unwashed semen specimen. Most impor-

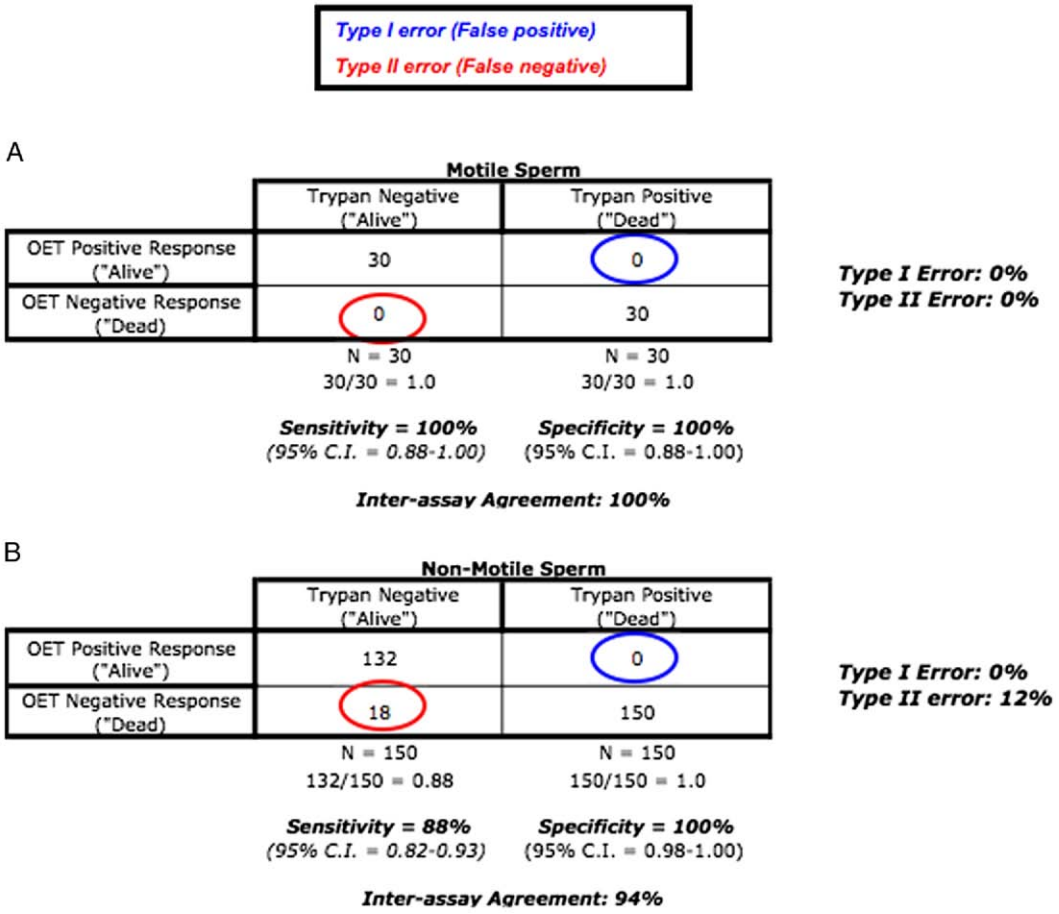

Figure 4. Individual sperm classified as alive or dead by trypan blue reference assay also underwent OET assay and were classified as alive with positive OET response or dead with negative response. For motile and nonmotile sperm OET type I error was 0 and type II error was 12%. *A,* 5 sperm per subject for total of 30 were classified per assay. OET was 100% sensitive and specific vs trypan blue assay. *B,* 25 motile and 25 nonmotile sperm were assayed in each of 6 subjects for total of 150 sperm each. OET was 88% sensitive and 100% specific vs trypan blue assay.

tantly the OET assay does not require direct physical contact with sperm since sperm remain suspended in solution at all times, nor does it require exposing sperm to potentially harmful chemical agents. OET allows individual sperm to be manipulated in space, providing a valuable means of sperm sorting and retrieving individual assayed sperm for ICSI or scientific analysis.

Compared to the trypan blue assay the mean sensitivity and specificity of OET was 88% and 100%, respectively. Type I error (false-positive, that is misclassification of a dead sperm as alive) was 0%. Type II error (false-negative, that is misclassification of a live sperm as dead) occurred for only 12% of nonmotile sperm classified as alive by trypan blue assay (classified as dead by OET). We speculate that this 12% fraction was actually misclassified by the trypan blue assay and not by OET. Vital dye exclusion tests such as trypan blue are known to have up to an 18% baseline false-positive rate.[21] Nonviable cells in the process of dying may still partially exclude trypan blue dye and, thus, yield a false result.[22,23] Low cell metabolic activity upon trypan dye exposure can delay dye uptake into a dying cell, such that OET correctly classifies viability sooner than the trypan assay. Others suggested that false trypan assay results occur due to vital dye-protein complexes that form outside the cell and preclude dye entry into a nonviable cell.[21] A substance from the ejaculate may collect around these spermatozoa and serve as a barrier to the cell wall, preventing inward diffusion of the trypan blue dye. To adjudicate the disagreement between OET and trypan assays independent secondary order assays of sperm viability are needed.

OET addresses the key limitations of current sperm selection technologies. Most importantly OET does not require that sperm die during the assay. Rather, assayed sperm appear to remain viable and can potentially be used for ICSI. Also, the OET assay operates at the level of individual sperm, which makes it more ideal for ICSI. Lastly, due to its ability to manipulate sperm in space OET provides a

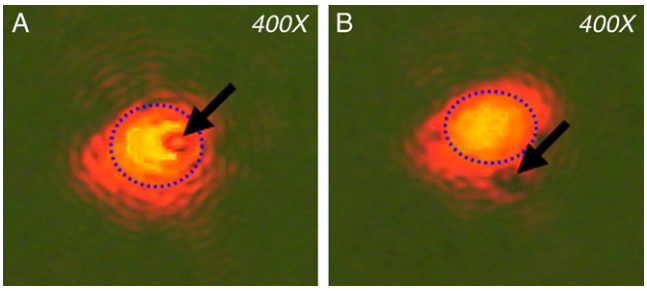

Figure 5. OET field can be created in any shape, pattern or size. *A* and *B*, pinpoint OET field (outlined white-pink area) was projected on sperm sorting micro-chamber. Live trypan negative sperm were attracted to field center. Dead sperm were repulsed and only found outside/peripheral to field. Dead cells in more advanced state of death/decomposition responded neutrally (no response) (data not shown). Pink-orange area surrounding OET field corresponds to light reflection from OET light pattern and is not part of field. *A*, trypan negative (live) nonmotile sperm. *B*, trypan positive (dead) nonmotile sperm.

physical means to retrieve individual sperm immediately after assay.

Since OET is optically generated, it can be used to assay sperm more efficiently than fixed electrode DEP with an assay exposure time of less than 1.5 minutes per sperm. The time needed to assess large sperm samples can be minimized by increasing OET assay throughput. For example, as we previously described,[16] multiple OET fields can be simultaneously projected on the sorting field, allowing massively parallel manipulation and assay to be done.

A potential limitation of our study is that the OET and trypan assays could not be performed independently of each other to exclude the possibility of a confounding effect of trypan blue dye during OET. Ideally the OET assay would be done first to yield groups of nonmotile sperm classified by OET as alive and dead. The trypan blue assay would then be performed in the OET classified groups to assess assay agreement. However, due to manufacturing limitations the chip prototype that we used does not allow individual cells to be extracted from the chip after the OET assay since it is necessary to allow each assay to be done independently of each other. We recently designed sorting chips with micro-ports, through which cells can be delivered to and retrieved from the OET field individually or in groups. These chips are pending manufacture. For example, a viable nonmotile sperm could be individually isolated from the remaining specimen using the operator guided OET field to pull the sperm toward the retrieval port, and retrieved for immediate use in ICSI.

Previous studies suggest that trypan blue dye has no independent effect on the OET assay response.[16] When OET was used to sort viable from nonviable human B lymphocyte cells, the response in lymphocytes known to be viable was identical to that in trypan negative lymphocytes. Similarly lymphocytes known to be dead but not exposed to trypan blue had a response identical to that of trypan positive lymphocytes.

When desired, OET assay throughput can be increased in real time by enlarging the size of the projected OET field and/or projecting multiple OET fields on the sorting field at once for massively parallel cell sorting.[16] These features provide higher order analysis and sorting functions that require a specialized manufacturing process and were not available for the prototype chip design used in this initial study. Also, although it is now more common to use washed specimens in the clinical setting, the OET assay could obviate the need for semen washing/centrifugation before retrieval since sperm viability can be individually and rapidly assayed.

In the current study we used semen samples from only relatively young, presumably fertile men. Sperm from older infertile men would more closely resemble the target (infertile) patient population and should be the focus of further study. Future studies should also compare the ability of the OET assay to predict sperm viability to that of alternative assays that measure sperm quality indexes, such as zona pellucida/hemizona binding, acrosome reactions and sperm head DNA decondensation assays.[3,24] Although we anticipate a low risk of DNA damage, potential harm to sperm by OET must be rigorously assessed. The net energy in J/m^2 delivered to the cell surface from our OET device is 5 orders of magnitude lower than the threshold value for light induced damage in human lymphoblasts.[25] While we detected no obvious gross evidence of harm caused by OET, more definitive studies are needed to assess OET exposure safety thresholds and potential mechanisms of injury to sperm structure and DNA.

CONCLUSIONS

The OET sperm assay and sorting platform presented improve on the limitations of currently available sperm selection technologies by allowing individual sperm to be assayed for viability and retrieved independent of motility. Since sperm motility is often not present or reliably discernible, the OET assay described could potentially improve ICSI outcomes.

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