Systems Biology in Reproductive Medicine

ISSN: 1939-6368 (Print) 1939-6376 (Online) Journal homepage: https://www.tandfonline.com/loi/iaan20

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**To cite this article:** Marcello Rubessa, Mikhail E. Kandel, Sierra Schreiber, Sasha Meyers, Douglas H. Beck, Gabriel Popescu & Matthew B. Wheeler (2020) Morphometric analysis of sperm used for IVP by three different separation methods with spatial light interference microscopy, Systems Biology in Reproductive Medicine, 66:1, 26-36, DOI: 10.1080/19396368.2019.1701139

**To link to this article:** https://doi.org/10.1080/19396368.2019.1701139

SYSTEMS BIOLOGY IN REPRODUCTIVE MEDICINE
2020, VOL. 66, NO. 1, 26–36
https://doi.org/10.1080/19396368.2019.1701139

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RESEARCH ARTICLE



# Morphometric analysis of sperm used for IVP by three different separation methods with spatial light interference microscopy

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**ABSTRACT**

The goal of this study was to characterize sperm populations resulting from three different methods of sperm selection used for bovine *in vitro* fertilization. We compared sperm selection with discontinuous Percoll gradients, Swim-Up, and electro-channel. Spatial light interference microscopy (SLIM) was used to evaluate the morphology of the spermatozoa and computer-assisted semen analysis (CASA) was used to evaluate the motility behavior of the sperm. Using these two technologies, we analyzed morphometric parameters and the kinetic (motility) patterns of frozen-thawed Holstein bull spermatozoa after sperm selection. For the first time, we have shown that these methods used to select viable spermatozoa for *in vitro* fertilization (IVF) result in very different sperm subpopulations. Almost every parameter evaluated resulted in statistical differences between treatment groups. One novel observation was that the dry mass of the sperm head is heavier in spermatozoa selected with the electro-channel than in sperm selected by the other methods. These results show the potential of SLIM microscopy in reproductive biology.

**Abbreviations:** SLIM: spatial light interference microscopy; CASA: computer aided sperm analysis; IVF: in vitro fertilization; BSA: bovine serum albumin; QPI: quantitative phase imaging; IVEP: in vitro embryo production; IACUC: institutional animal care and use committee; CSS: Certified Semen Services; AI: artificial insemination; TALP: Tyrode's Albumin Lactate Pyruvate; MEC: medium for electro-channel; PDMS: polydimethylsiloxane; EC: electro-channel; TM, %: total motility; PM, %: progressive motility; RM, %: percentage of rapid sperm motility; VAP, μm/s: average path velocity; VSL, μm/s: straight-line velocity; VCL, μm/s: curvilinear velocity; ALH, μm: amplitude of lateral head displacement; BCF, Hz: beat cross frequency; STR, %: straightness; LIN, %: and linearity; GLS: generalized least squares; ANOVA: analysis of variance; LSD: Least Significant Difference; SPSS: Statistical Package for the Social Sciences; PCA: principal components analysis

**ARTICLE HISTORY**
Received 20 June 2019
Revised 21 October 2019
Accepted 22 October 2019

**KEYWORDS**
SLIM; sperm; morphometry; CASA; bovine; IVF

## Introduction

Frozen-thawed bull semen has been used extensively for *in vitro* fertilization (IVF). It is well known that some sperm are killed or damaged during the freezing and thawing processes (Celeghini et al. 2008). These dead or damaged sperm interfere with fertilization during IVF. Furthermore, it is necessary to separate motile sperm from the other components (seminal plasma, extender, and/or undesired cells) that are contained in the freezing straw for optimal fertilization rates during IVF (Parrish J 1991; Parrish J. J. et al. 1995). Several techniques have been developed to recover a homogeneous population of highly motile sperm cells (Oliveira et al. 2011). However, not all separation methods are ideal, some are not fast, easy, or sufficiently economical for routine use in an IVF lab (Henkel and Schill 2003). Currently, the two most common methods to select motile spermatozoa are: discontinuous Percoll gradients and Swim-Up. For Percoll gradients, a sperm sample is placed on a high density medium, centrifuged, and the pellet obtained is enriched with highly motile spermatozoa (Parrish J. J. et al. 1995). The other sperm separation method, Swim-Up, is based on the self-movement of sperm in a column of medium containing bovine serum albumin (BSA), typically 0.6% (Parrish J. J. et al. 1995; Parrish John J., 2014). Thawed semen is placed at the bottom of a centrifuge tube containing the Swim-Up medium and motile sperm separate themselves from the other

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components during an incubation period (~1 hr). The semen extender is denser than the medium in the column allowing the most motile sperm to swim out of the extender and up the column. To augment these two methods, several new methods have been designed to select sperm for IVF (Ishijima et al. 1991; Valcárcel et al. 1996; Sterzik et al. 1998). In our laboratory, we designed a method to select viable spermatozoa using electromagnetic fields (Rubessa et al. 2016). This technique takes advantage of one physiological characteristic of high quality sperm, the electric charge. Mature bull sperm have a zeta potential (Ishijima et al. 1991) of −16 to −20 mV (Rubessa et al. 2016). It has been shown previously that the highest quality sperm are also the most electronegative (Chan et al. 2006). The goal of all of the listed methods is to select the 'best' sperm population for IVF. Morphology and motility are the primary characteristics being selected for but other characteristics are not well known and few studies have been conducted to identify such characteristics (Somfai et al. 2002; Arias et al. 2017).

To analyze the morphometry parameters (dimensions and dry mass) of the sperm head, middle piece, and tail we chose Spatial Light Interference Microscopy (SLIM), a white-light, common path method. This method is capable of imaging unlabeled cells and we previously used it to image bovine spermatozoa (Liu et al. 2018). To study the motility (kinetic) parameters (VAP, VSL, ALH, BCF, STR, LIN, PM%, RM% and TM%) of sperm we used computer-assisted sperm analysis (CASA). The objective of the present study was to evaluate the characteristics of three sperm populations selected after exposure to: 1) a discontinuous Percoll gradient, 2) Swim-Up or 3) electro-channel separation protocols.

## Results

The results reported in Tables 1 and 2 show the mean and standard deviation for each parameter. Using SLIM, we have been able to analyze the morphometric parameters of all spermatozoa including the dry mass weight inside the head and the middle piece of the spermatozoa (Figure 1). The results in Figures 2 and 3 show that the subpopulations created by the different sperm separation methods are completely different. Interestingly, the electro-channel group is different ($P < 0.01$) in all morphometric parameters measured compared with Swim-Up group (Table 1), but it is not significantly different from the Percoll group when comparing the length of the head or the tail. Between Swim-Up and Percoll treatments, the head length and midpiece length are not different. The remaining parameters (not described above) are significantly different ($P < 0.01$) or ($P > 0.05$) between treatments (Table 1). The dry mass

**Table 1.** The morphometric parameter measurements for bull sperm acquired with the SLIM microscope.

| Parameters | ElectroCh | Percoll Gradient | Swim-Up |
|---|---|---|---|
| Head Length | $9.26 \pm 0.39^{A}$ | $9.43 \pm 0.43^{A}$ | $9.37 \pm 0.47^{B}$ |
| Head Width | $4.32 \pm 0.36^{A}$ | $4.44 \pm 0.33^{B}$ | $4.57 \pm 0.37^{C}$ |
| Acrosome Length | $1.42 \pm 0.20^{A}$ | $1.83 \pm 0.45^{B}$ | $1.59 \pm 0.51^{C}$ |
| Acrosome Width | $3.63 \pm 0.34^{A}$ | $4.09 \pm 0.35^{B}$ | $4.22 \pm 0.40^{C}$ |
| Midpiece Length | $14.24 \pm 0.84^{A}$ | $13.98 \pm 0.70^{B}$ | $14.02 \pm 0.78^{B}$ |
| Tail Length (include midpiece) | $63.73 \pm 3.54^{A}$ | $64.28 \pm 2.95^{A}$ | $65.00 \pm 3.78^{B}$ |
| Cell Dry Mass Midpiece (pg) | $9.75 \pm 0.12^{A}$ | $6.27 \pm 0.09^{B}$ | $5.97 \pm 0.10^{C}$ |
| Cell Dry Mass Head (pg) | $5.69 \pm 0.09^{A}$ | $4.07 \pm 0.06^{Ba}$ | $4.30 \pm 0.07^{Bb}$ |
| Total Dry Mass (Head + Mid Piece) | $15.45 \pm 0.19^{A}$ | $10.35 \pm 0.13^{Ba}$ | $10.27 \pm 0.16^{Bb}$ |

The values acquired with the SLIM are in micrometers (μm) and pico-grams (pg). ElectroCh = Electro-Channel, Percoll Gradient = Discontinuous Percoll Gradient, Swim-Up = Swim-Up. $^{ABC}$differ significantly ($P < 0.01$); $^{abc}$differ significantly ($P < 0.05$) within the groups. Values are mean and standard deviation.

**Table 2.** Sperm kinetics values for bull sperm acquired with CASA.

| Parameters | ElectroCh | Percoll Gradient | Swim-Up |
|---|---|---|---|
| VAP | $92.00 \pm 10.31^{A}$ | $136.68 \pm 43.05^{B}$ | $81.70 \pm 6.52^{C}$ |
| VSL | $77.52 \pm 11.47^{A}$ | $125.76 \pm 47.03^{B}$ | $48.00 \pm 9.87^{C}$ |
| VCL | $169.06 \pm 20.32^{A}$ | $209.85 \pm 47.63^{B}$ | $167.84 \pm 15.82^{A}$ |
| ALH | $8.50 \pm 0.95^{A}$ | $7.36 \pm 0.48^{B}$ | $9.21 \pm 0.71^{C}$ |
| BCF | $27.32 \pm 6.79^{A}$ | $38.60 \pm 6.23^{B}$ | $19.83 \pm 1.30^{C}$ |
| STR | $80.60 \pm 4.04^{A}$ | $87.44 \pm 6.53^{B}$ | $61.07 \pm 4.47^{C}$ |
| LIN | $44.80 \pm 2.93^{A}$ | $57.70 \pm 6.23^{B}$ | $31.04 \pm 2.68^{C}$ |
| TM % | $64.60 \pm 25.23^{A}$ | $61.18 \pm 26.00^{A}$ | $71.40 \pm 6.70^{B}$ |
| PM % | $48.00 \pm 24.52^{A}$ | $44.45 \pm 17.68^{B}$ | $20.08 \pm 5.36^{C}$ |
| RM % | $54.20 \pm 26.89^{A}$ | $51.62 \pm 20.95^{B}$ | $59.44 \pm 4.02^{C}$ |

The CASA parameters measured were: Average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %). ElectroCh = Electro-Channel, Percoll Gradient = Discontinuous Percoll Gradient, Swim-Up = Swim-Up. $^{ABC}$differ significantly ($P < 0.01$); $^{abc}$differ significantly ($P < 0.05$) within the groups. Values are mean and standard deviation.

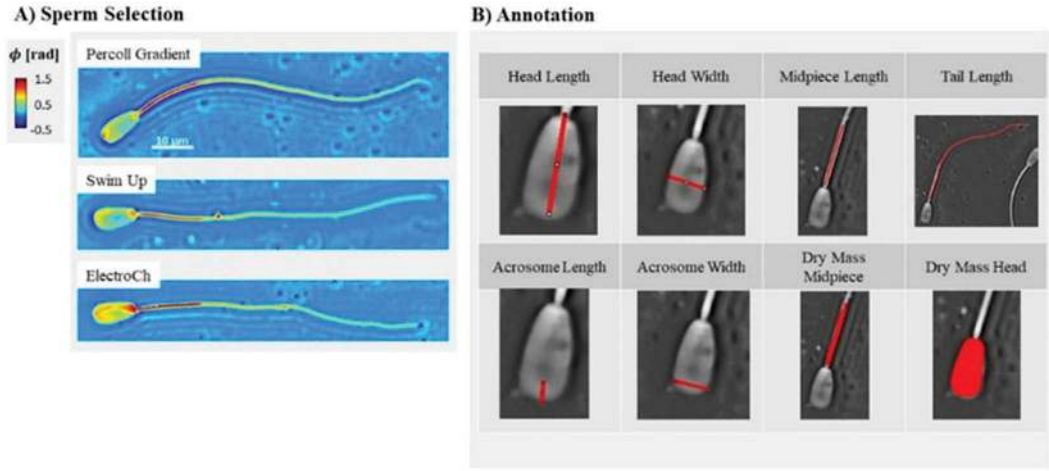


**Figure 1.** SLIM density images (Panel A) of sperm selected by the three separation methods; discontinuous percoll gradient (top), Swim-Up (middle), and electro-channel (bottom). SLIM images depicting the sperm measurements performed on the individual sperm (Panel B).

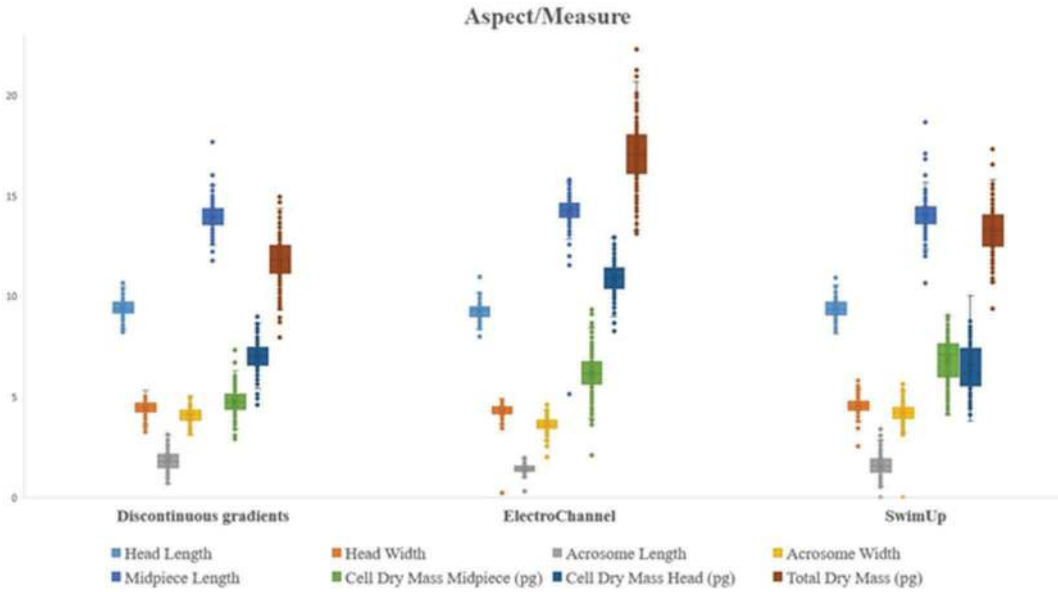


**Figure 2.** Population distribution of sperm separated by the three methods (discontinuous percoll gradient, electro-channel, and swim-up). Measurements of head, acrosome, and middle piece (μm) dimensions.

of midpiece was different ($P < 0.01$) between all treatments with the electro-channel treatment having the heaviest midpiece. The sperm heads in the electro-channel treatment had a higher ($P < 0.01$) dry mass than the sperm heads of the other 2 treatments. When comparing the total dry mass (head + midpiece), we found that the electro-channel treated sperm was heavier ($P < 0.01$) than sperm from either the Swim-Up or discontinuous Percoll treatments. In addition, sperm from the Percoll treatment had a greater total dry mass ($P > 0.05$) than Swim-Up-treated sperm (Figure 4).

It was interesting to note when we analyzed the CASA results, only two parameters were not statistically different, the VCL and the TM% (Table 2, Figure 5). In these cases when we examined the VCL, the Swim-Up and electro-channel treatments had similar results, as did the Percoll and electro-channel treatments for TM%. All the other parameters (VAP, VSL, ALH, BCF, STR, LIN) are different ($P < 0.01$) in the three subpopulations. When we analyzed the other motility parameters, we found higher ($P < 0.01$) total motility in the Swim-Up group compared with the others but lower ($P < 0.01$) progressive motility

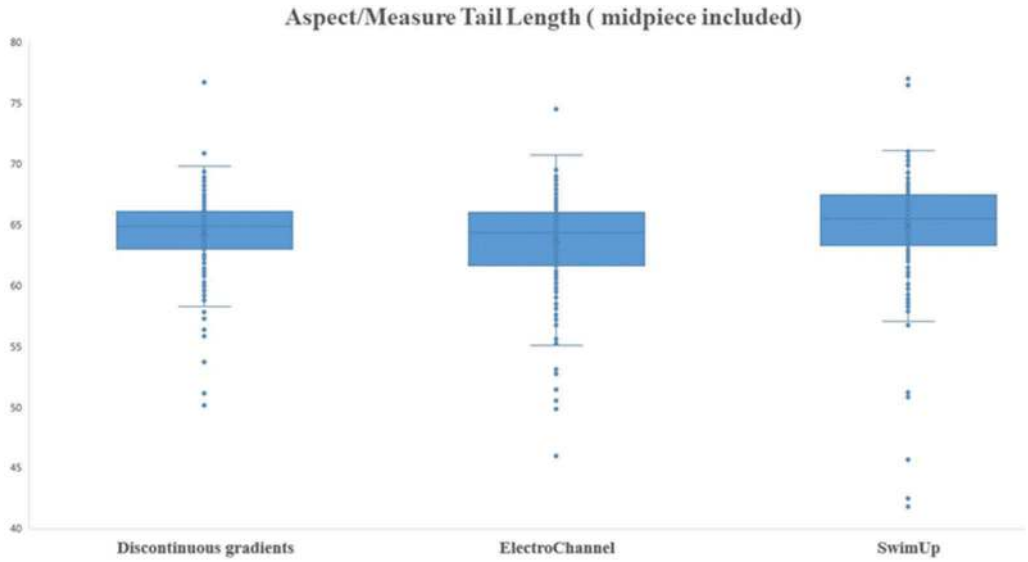


**Figure 3.** Population distribution of sperm separated by the three methods (discontinuous percoll gradient, electro-channel, and swim-up). Measurement of the tail (μm) length.

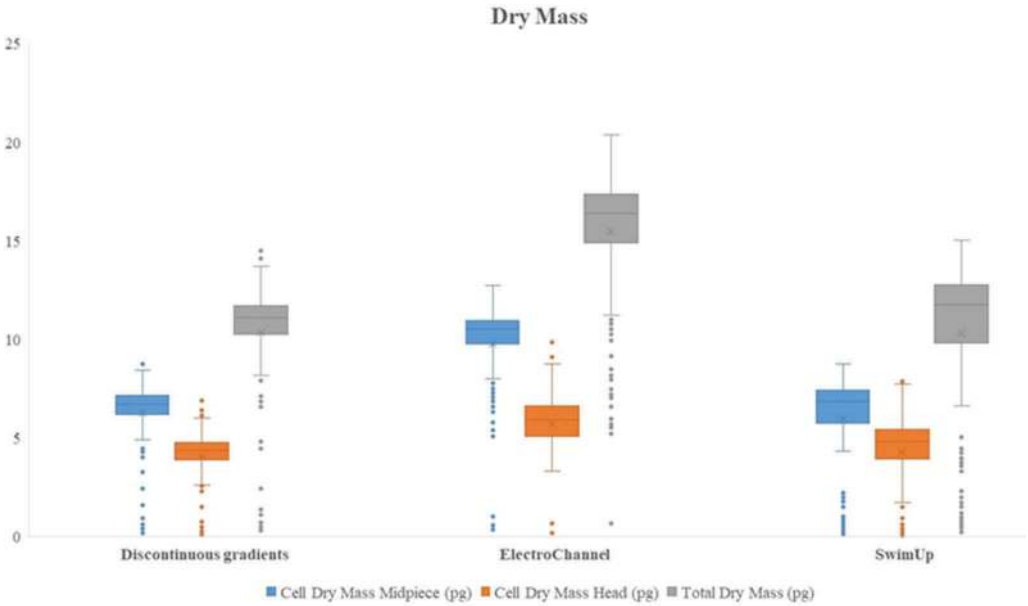


**Figure 4.** Population distribution of sperm separated by the three methods (discontinuous percoll gradient, electro-channel, and swim-up). Measurements of the head, midpiece, and total dry mass (pg).

than the other 2 treatments (Table 2). The rapid motility was different between all three treatments with the Swim-Up treatment being the highest ($P < 0.01$) (Table 2, Figure 6). There was no difference found in total motility between Percoll and electro-channel treatments.

There is substantial variability in the kinetic data (VAP, VSL, ALH, BCF, STR, LIN, PM%, RM% and TM%) between the three different sperm separation systems. One explanation is that we report the means and standard deviation for these measures. Typically, CASA kinetic data is reported as mean and standard error of the mean (Nongbua et al. 2018). Papers that use CASA analysis that also report mean and standard deviations see similar variability as described here and show significant differences between bulls examined (Nagy et al. 2015). In the present study, we used pooled semen from 3 bulls, which could explain the large variability in the CASA kinetic parameters observed.

The final analysis completed was a principal component analysis (PCA) that evaluates the trend among the

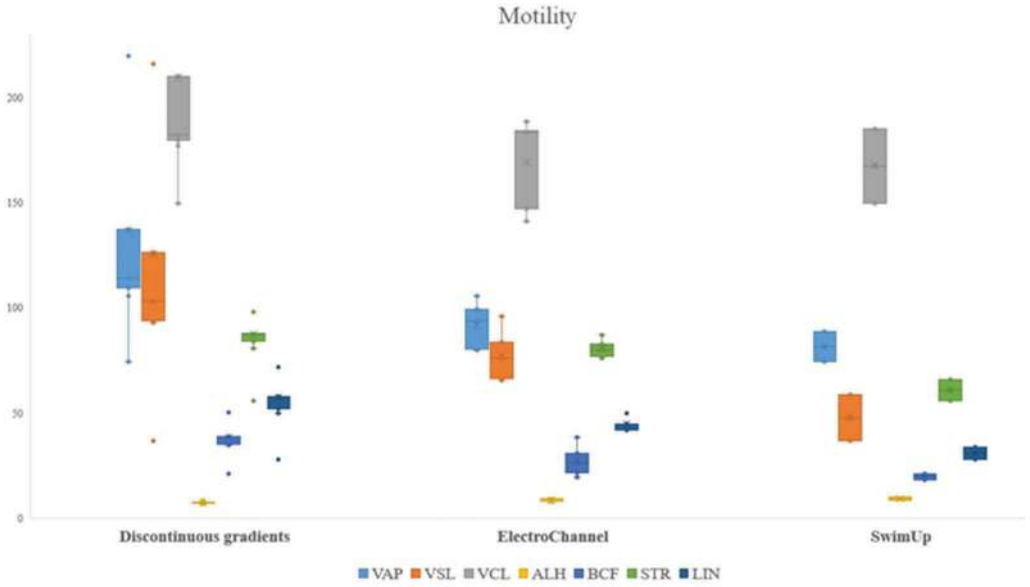


**Figure 5.** Population distribution of sperm separated by the three methods (discontinuous percoll gradient, electro-channel, and swim-up). Measurements of the CASA parameters: Average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %).

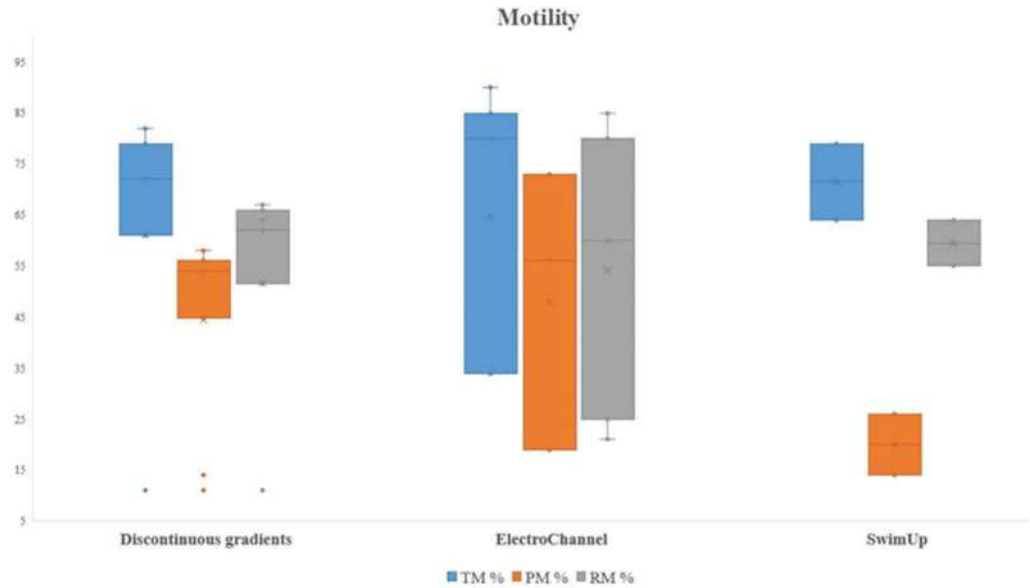


**Figure 6.** Population distribution of sperm separated by the three methods (discontinuous percoll gradient, electro-channel, and swim-up). Measurement of the CASA parameters: Total motility (TM, %), progressive motility (PM, %), and rapid motility (RM, %).

variables. PCA is a data mining technique that reduces the redundancy among variables creating uncorrelated features called principal components with minimum information loss (Milewska et al. 2014). PCA keeps as much variability in the data as possible, and enables visualization of observations (Milewska et al. 2014). When we evaluated the morphometry data, we found that the Percoll and Swim-Up groups had the same trend while the electro-channel group had a different trend (Figure 7). When we examined the motility results with PCA the results showed three different behaviors, one from each of the separation methods (Figure 8). It is interesting to note, that while PCA of motility reduced the variable to three plots, the

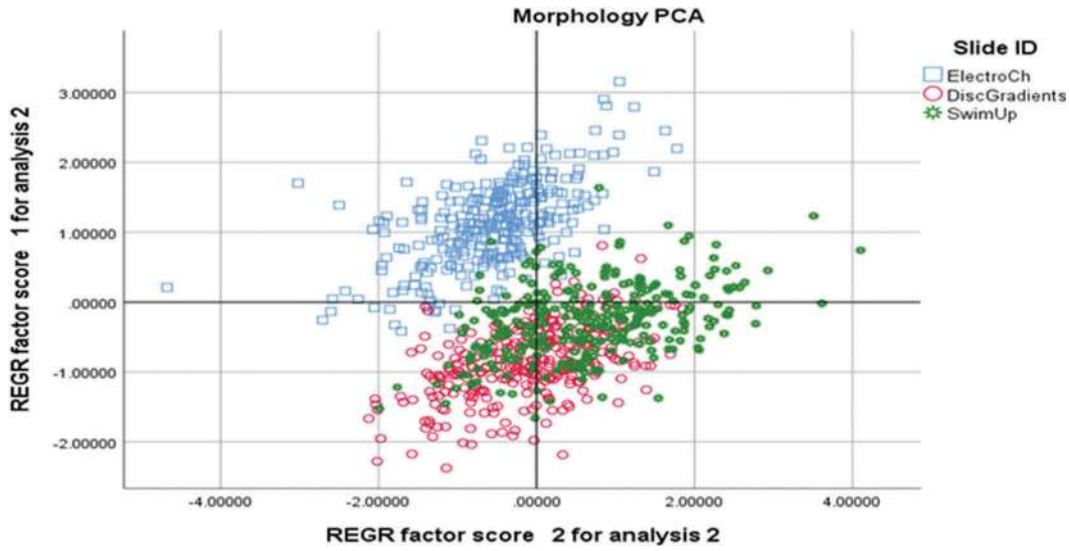


**Figure 7.** Principal component analysis in 2D: Plot of the morphometry between discontinuous Percoll Gradient, Swim-Up, And Electro-Channel.

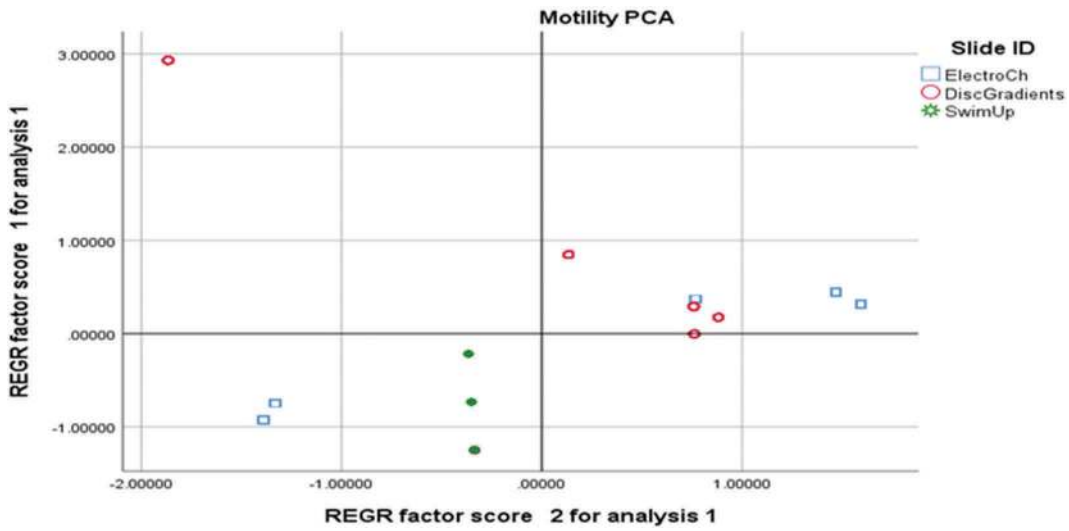


**Figure 8.** Principal component analysis in 2D: Plot of the motility between discontinuous Percoll gradient, swim-up, and electro-channel.

morphology analysis did not reduce the number of variables.

When we performed correlation analysis on a portion of the morphometry and motility parameters, we found some interesting correlations (Figure 9). We observed little correlation between total motility (TM %) and the morphometry parameters (tail length, mid-piece dry mass, or head dry mass). The same relationship was observed between rapid motility (RM%) and those same morphometry parameters. In contrast, we observed a positive correlation (r = 0.253) between the progressive motility and the dry mass of the middle piece, the dry mass of the head (r = .137), and of the total dry mass (r = .226, data not shown in Figure 9). The length of the tail had no significant correlation with any of the motility parameters. The three motility parameters (PM%, RM%,and TM%) all showed strong positive correlations, PM% to TM% (r = .619), PM% to RM% (r = .676), and RM% to TM% (r = .976) (Figure 9).

## Discussion

Sperm treatment protocols are routinely used to separate viable sperm from the diluent and dead cells in frozen–thawed semen. There are several techniques for the sperm separation such as Swim-Up, discontinuous

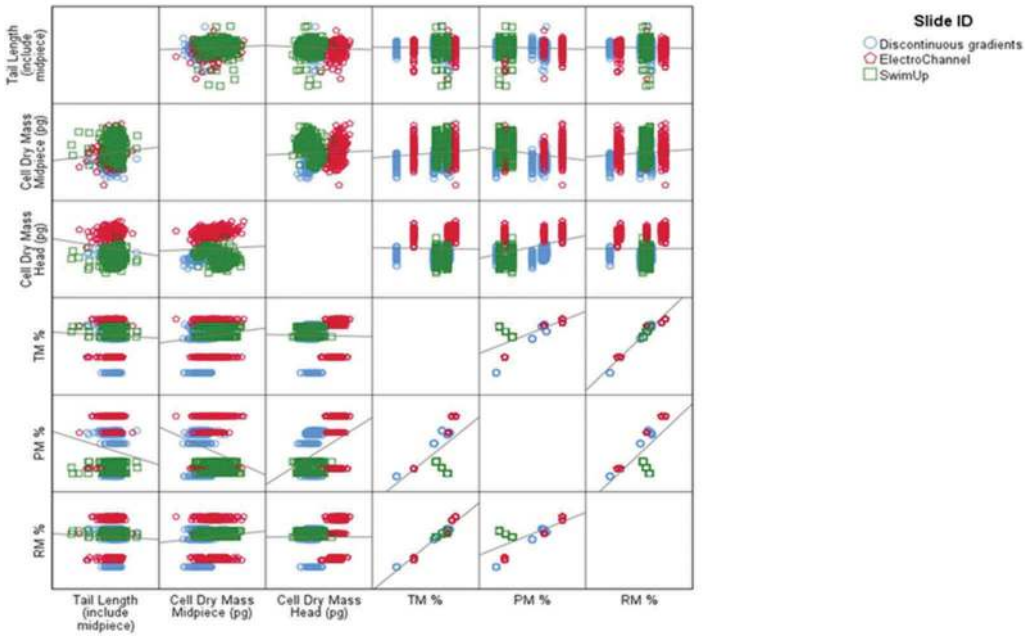


**Figure 9.** Pearson Correlation scatter plot for sperm motility, morphometry, and dry mass parameters. Correlations of total motility (TM, %), progressive motility (PM, %), rapid sperm motility (RM, %) *vs*. tail length and dry mass of sperm middle piece and head. Trend lines were fitted to the data to illustrate the trends.

Percoll gradients, Sephadex (Valcárcel et al. 1996) and glass wool filtration (Sterzik et al. 1998) routinely used in human and bovine IVF. Numerous studies that compare the morphology of sperm after different separation methods all have the same limitation, the sperm were evaluated after staining. Previous studies have demonstrated modification of the morphology of sperm after staining (Banaszewska et al. 2015; Kondracki et al. 2017; Maree et al. 2010). Further, the majority of these studies were focused on the sperm motility and few analyzed the morphology. This creates a knowledge gap in the IVF field. The results obtained in the present study confirmed that each method selects different sperm subpopulations, this is in agreement with other reports (García-Herreros and Leal 2014). The first interesting result was unexpected, the total weight of the sperm selected with Percoll was lighter than those separated by the electro-channel. This result is due to the fact that the dry mass of the midpiece of the electro-channel sperm was heavier (9.76 ± 0.12 pg *vs* 6.27 ± 0.09 pg) than the Percoll separated sperm. One possible explanation for the heavier dry mass of the sperm in the electro-channel is the high negative charge of sperm, which causes them to move toward the cathode (Zeng et al. 1995). This means that electro-channel selected sperm may have a higher negative charge than the non-selected sperm and this is likely due to a larger total dry mass (head and midpiece). Despite having a heavier head, the sperm population selected by electro-channel has a shorter length compared with the other two groups. It is important to highlight the acrosome size in the electro-channel population. The acrosome is smaller in the electro-channel group than the other two groups. We could speculate that the magnetic field activates the acrosome reaction in some sperm. It is known that the acrosome reaction is voltage-dependent through the potassium and calcium channels (Zeng et al. 1995). Furthermore, our results confirm a previous study that showed that the integrity of the acrosome was higher in the sperm population processed by Percoll gradients than Swim-Up (Somfai et al. 2002). Further analysis is needed to determine if the heavier sperm head is meaningful for IVF and the resulting embryos produced.

The differences in size and weight of sperm subpopulations is not surprising as the three methods used to separate the sperm use different physical principles. The discontinuous Percoll gradients separate sperm according to buoyant density so heavier sperm are selected. In the electro-channel the sperm with the most negative charge, the heavier sperm (from the present results), are selected by constant electric field. The sperm selected by the Swim-Up method are are the most motile and displace less fluid in the BSA column, and, therefore, travel the furthest in that column. The Swim-Up sperm are intermediate in head weight but

have the longest tails. Again, not surprising Swim-Up selects for sperm that swim the best and a longer tail likely facilitates this swimming. Future studies will focus on modeling of differences observed in sperm as a function of the type of sperm separation method.

The results showed a higher progressive motility for Percoll group compared to the others. These results confirmed the reports of a previous study where the authors showed similar differences between Percoll or Bovipure™ gradients and Swim-Up separation (Arias et al. 2017). Instead, the total and rapid motility was statistically different only between the Swim-Up and the other treatments ($P < 0.01$). All the other motility parameters were statistically different between the groups, with only the VCL between the electro-channel and Swim-Up showing no difference. The VCL in a previous paper was identified as an important parameter to evaluate the motility (Farooq et al. 2017). That paper and our results both showed a higher VCL value for the Percoll group compared the other two groups.

One of the results obtained when correlations between motility and the morphometry were performed, was the direct correlation between morphometric parameters and progressive motility (Figure 9). The analysis showed a positive correlation ($r = 0.254$, $r = 0.137$, and $r = 0.226$ respectively) between the progressive motility and the dry mass (head, middle piece, and total dry mass). We also confirmed that the motility is indirectly correlated with the length of the tail (Figure 9). In contrast, there were no statistical correlations between any of the morphometric parameters and the other two motility markers (total motility and rapid motility).

## Conclusion

Using SLIM microscopy, we were able to analyze sperm morphometry without morphometric modifications. This is possible because sperm were analyzed without staining or labeling. Our results showed that each method of sperm selection chooses a different sperm sub-population (Figure 10). Our results showed almost all of the parameters measured were significantly different. The present study provides additional evidence that confirms the importance of the specific morphometry of different parts of the sperm cell anatomy. For the first time we have shown how the dry mass (head, middle piece, and total dry mass) influences (directly or indirectly) the most common parameters used to evaluate motility. These results highlight the importance of picking the optimal method of sperm selection for your goals. The SLIM technology has enabled the collection of these results and we believe that SLIM will become a fundamental method for sperm evaluation for assisted reproduction and the study of basic reproductive biology.

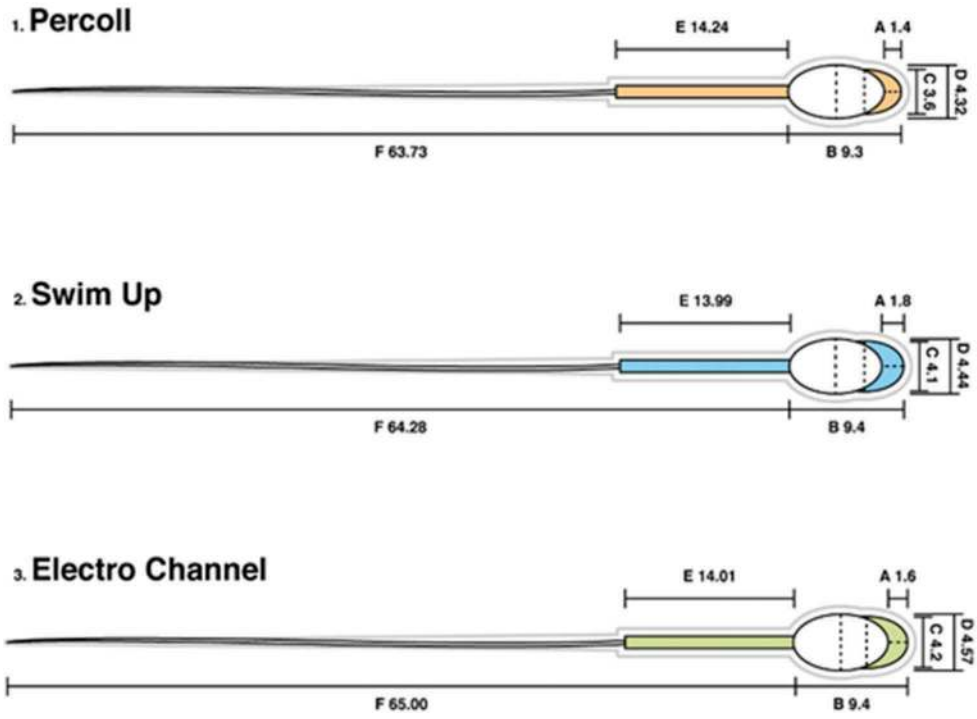


**Figure 10.** Schematic representation of the morphometry of the sperm subpopulations selected by three separation methods: Percoll (1), swim up (2), and electro-channel (3). Schematics include length and width of both head and acrosome, and length of middle piece and tail. (Illustration courtesy of Dr. Costantino Costantini).

## Material and methods

### Experimental design

Three different bulls were used for this study, all of which were previously tested for *in vitro* embryo production (IVEP). IACUC approval was not required by the University of Illinois IACUC since only previously frozen sperm samples were purchased and used for this research. Semen was collected, extended, and frozen according to standard industry procedures (Certified Semen Services (CSS) protocols) at Interglobe Genetics AI Center (Pontiac, IL). Six straws per replicate were thawed (two for each bull); all straws were collected (pooled) in the same tube and then divided for use in the individual separation treatments. Three different sperm selection methods were used: Swim-Up, discontinuous Percoll gradients, and electro-channel. After each sperm selection procedure, we took 3 µl from each sperm pool for CASA analysis and another 10 µl were put on a slide and air dried for SLIM analysis (Liu et al. 2018). The experiment was replicated 6 times, each SLIM replicate read 50 spermatozoa cells. A total of 300 sperm per group were evaluated by SLIM.

### Sperm selection methods

Semen straws from three bulls were removed from the liquid nitrogen tank and thawed in a water bath at 37°C for 40 seconds. After thawing, the semen was processed with each of the three protocols: discontinuous Percoll gradient (45% to 90%), Swim-Up, and electro-channel.

### Discontinuous percoll gradients

Discontinuous Percoll gradients were prepared by combining Sperm-TALP (Tyrode's Albumin Lactate Pyruvate) and ISO-Percoll. Sperm-TALP-basic-medium (Sattar et al. 2011), was supplemented with pyruvic acid (Sigma-Aldrich, St. Louis, MO) and gentamycin sulfate (Sigma-Aldrich, St. Louis, MO). ISO-Percoll was made by combining sodium bicarbonate with Earle's Balanced Salt Solution (EBSS) (Thermo Fisher Scientific, Waltham, MA) and vortexing in a tube, then combining with Percoll® (Sigma-Aldrich, St. Louis, MO). The pH was adjusted to 7.4. ISO-Percoll and Sperm-TALP were combined to create 45% and 90% mixture gradients (45% on the top of 90%). The osmolarity of the ISO-Percoll was measured at 297–303 mOSM by freezing point depression. Semen was slowly pipetted down the side of the tube to create a third layer on top of the 45% gradient and then centrifuged at 460 x g for 25 minutes. The supernatant was discarded, and the pellet was washed in one milliliter of Sperm-TALP and centrifuged at 250 x g for 10 minutes. The supernatant was again discarded, and the pellet was suspended in one milliliter of Sperm-TALP and centrifuged at 170 x g for 10 minutes. The supernatant was removed and 50 µl of Sperm-TALP was added to the pellet.

### Swim-up

The sample of thawed semen was layered carefully under 1 ml of equilibrated sperm-TALP medium with 6 mg of BSA (Bovine Serum Albumin, Fraction V fatty acid-free, Sigma-Aldrich, St. Louis, MO) per ml in a 5 ml round bottom centrifuge tube. After loading, the tube was placed in an incubator at 39°C for 1 hour. After incubation, 400 µL of the upper fraction (containing the selected sperm) was collected, placed in a tube, and centrifuged for 10 min at 160 x g (Somfai et al. 2002).

### Electro-channel (EC)

The medium for electro-channel (MEC) contained: NaCl 98.8 mM; KCl 3 mM; $NaH_2PO_4$ 0.35 mM; $CaCl_2.2H_2O$;
$MgCl_2.6H2O$; $NaHCO_3$ 25 mM; HEPES 10 mM, plus 10 mg/mL of BSA. The pH and the osmolarity were respectively 7.4 and 280 mOSM. The device was made from of polydimethylsiloxane (PDMS) as previous described (Rubessa et al. 2016). After thawing, semen was centrifuged for 5 min at 160 x g and 10 µL of pellet were positioned in the EC, containing 1 mL of MEC, at the center of the electro-channel. The electric charge used was 10 volts for 10 minutes. After incubation, 100 µl of MEC from the cathode was collected and centrifuged 5 min at 160 x g.

### Computer-assisted semen analysis (CASA)

A CASA IVOS system (Hamilton Thorne, Beverly, MA, USA) was used to measure total motility, rapid motility, and progressive motility of sperm. The software was set per manufacturer's recommendations for the assessment of motility characteristics of bovine bull spermatozoa as follows: frames acquired: 30; frame rate: 60 Hz/s; minimum contrast for cell detection: 80; minimum cell size: 5 pix; Progressive VAP 50 µ/s; straightness threshold: 70%; slow VAP cut off: 30 µ/s; slow VSL cutoff: 15 µ/s; and magnification factor 1.92. For each sample, 3 µl was removed, and 10 microscopic fields were analyzed. The image knob was adjusted until the sperm were clearly visible on the monitor. The following sperm kinetic parameters were analyzed: total motility (TM, %), progressive

motility (PM, %), percentage of rapid sperm motility (RM, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %).

### SLIM analysis

Ten microliters of sperm were added to a glass slide and the sample was drawn across the slide to spread it evenly. These slides were air-dried and stored at 4°C until analysis. Images were taken of the slides using the SLIM quantitative phase imaging (QPI) instrument described in (Liu et al. 2018). Quantitative phase imaging (QPI) (Popescu 2011) is a label-free, nondestructive imaging modality that has important biomedical applications (for a recent review, see (Park et al. 2018)). QPI techniques yield a phase rather than an intensity map, which allows for quantitative measurements on transparent specimens, such as unlabelled cells. In reproductive research, such systems have found fertile ground in applications characterizing whole embryos (Warger et al. 2007; Nguyen et al. 2017) as well as sperm (Balberg et al. 2017; Lee et al. 2018). For the present study we chose spatial light interference microscopy (SLIM), which is highly sensitive in both space and in time. Because SLIM uses white light, which averages speckles, and common path interferometric geometry, which insures phase stability, it is highly sensitive (Kandel et al. 2017). SLIM has been used recently to characterized the topography and refractometry of sperm (Liu et al. 2018; Rubessa et al. 2019). Images were manually segmented using the ROI feature in ImageJ (NIH, Bethesda, MD). To eliminate between-researcher variation, all images were manually annotated by the operator using the ROI feature in ImageJ (Figure 1). We found all parameters circumscribed by sharp refractive index contrasts, except the acrosome, which in 2D images appears as a dense bump at the tip of the sperm head. As outlined in (Kandel et al. 2018), dry-mass was calculated as a scaled sum of the halo-corrected phase values within a selection (ImageJ's Integrated Density measure, NIH, Bethesda, MD). In-line with common selection criteria, we chose to analyze all imaged spermatozoa that displayed intact acrosomes.

## Statistical analysis

A total of 902 bull sperm were evaluated. The values obtained, for morphometry and motility, were analyzed using generalized least squares (GLS) ANOVA analysis using *post hoc* analysis with LSD correction.

In the second phase analysis of our results, we ran a Pearson correlation (two-tailed), because we wanted to evaluate the relationship between morphometric results and the kinetic (motility) results. For this analysis, we evaluated the correlation between morphometry parameters (head dry mass and midpiece dry mass, and tail length), and the motility parameters (total, progressive and rapid motility) with SPSS (SPSS version 25. IBM, New York). In both analyses the P value was set at $P < 0.01$. We performed a principal component analysis (PCA) to characterize the variation in the morphometric and kinetic parameters using SPSS (SPSS version 25. IBM, New York). PCA allows for the examination of a small number of variables by reducing the redundancy among those variables and revealing linear relationships between the variables.

## Acknowledgments

The authors would like to thank Molly Sermersheim and Sierra Alicia Long for careful reading of the manuscript. Furthermore, we would like to thank Dr. Costantino Costantini for his illustrations.

## Disclosure statement

G.P. has a financial interest in Phi Optics, Inc., a company developing quantitative phase imaging technology for materials and life science applications.

## Funding

This work was partially supported by the USDA Multistate Project W-4171 (MBW), the Ross Foundation (MBW, MR), and by National Science Foundation grant NRT-UtB 1735252 (MEK).

## Author Contributions

Experimental design: MR and MBW; Sample preparation: MR and MEK; SLIM imaging: SS; Manual segmentation on SLIM images: SS and SM; Data analysis: MR; MEK and DHB; Supervised project: GP, MBW; Wrote manuscript: MR and MBW.

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