

QUALITY COMPARISON OF THREE DISPOSABLE SPERM CELL COUNTING CHAMBERS

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DRAFT REPORT

ABSTRACT AND CONCLUSION

In this study various quality aspects of the CellVision, Leja, and MicroCell disposable sperm cell counting slides were investigated.

The height of the chambers was determined using interferometric spectrometry. The average heights of the Leja and MicroCell were very close, respectively 20.0 μm and 20.3 μm , whereas the CellVision was lower than specification (17.9 μm).

The accuracy and reliability of concentration and motility measurements using the chambers was investigated by analyzing various semen samples with the Ceros CASA system of Hamilton Thorne. Counting with the Leja and MicroCell slides results in very similar concentrations, which are also very close to the FACS outcomes (used as external standard). However, the standard deviation and range of the Leja measurements are smaller than of MicroCell. This is probably caused by a non-homogeneous distribution of sperm cells within the MicroCell chamber; at the end of the chamber 70% above the average value was found, whereas deviations within the Leja slide do not exceed 7.4%. For these reasons, Leja is the most reliable chamber for concentration determination.

The Leja slide has more advantages. First, less air bubbles are captured during chamber filling as compared to the MicroCell. Second, the highest motility values are measured in the Leja chamber. When using human sperm, motility in the MicroCell chamber is a few percent lower, but with the sensitive boar sperm, motilities in the MicroCell can be reduced by more than 50%. The CellVision chamber is extremely toxic for human, as well as for boar sperm. After 1 minute, hardly any motile cells can be detected anymore (for this reason, some experiments with the CellVision slide were omitted).

The results of this study show that there is something to choose for.

INTRODUCTION

Procedures in assisted reproduction are complex and have many variables. It is important to control each variable as much as possible in order to be in control of the total therapy. Accurate counting and motility measurements of sperm cells in a standardized manner is important, as it is essential for the clinical diagnosis and for the choice of treatment. This accuracy will improve by choosing a consistent and accurate counting slide and the proper counting method. Various counting chambers are used to determine sperm concentration. The 10 μm reusable Makler chamber, the hemocytometer of 100 μm , and various disposable slides with a height of 20 μm . For hygienic, efficiency and practical reasons, the demand for disposable slides is increasing. Furthermore, their quality is not influenced by long term usage induced damage, the cleaning procedure, or the manual assembly method.

In this study, we compare three disposable counting slides (CellVision, Leja, and MicroCell) for various aspects: chamber height, reliability of sperm concentration and motility determination, sperm distribution, toxicity, and filling behaviour. Concentration and motility were measured by a computer-assisted semen analysis (CASA) system for objectivity and practical reasons; CASA allows counting a large number of sperm cells, and to determine motility in a very short time span. To have an external calibration value, we included flow cytometry (FACS), as a new reference for the sperm concentration.

MATERIALS AND METHODS

General

The IVF laboratory provided nameless discarded semen samples. Both liquefied ejaculates and Pure Sperm-processed samples (in HTF/HSA) were used (as indicated in the text or legends).

Three disposable slides were evaluated: CellVision® (CellVision Company, Anthos, Heerhugowaard, The Netherlands, lot nr: 2002-5-0002, 23 jan 02), Leja SCAC (Leja Products,

Nieuw-Vennep, The Netherlands, SC20-01-C, batch nr: 020212) and MicroCell™ (Conception Technologies, USA, lot #: 6020221).

During the experiments, semen samples were kept at room temperature and slides were pre-heated to 37°C. Slides were loaded by capillary action using a negative displacement pipette. The sample was allowed to heat up to 37°C for 1 minute. Concentration and motility parameters were determined using the Hamilton Thorne CEROS using the program “Human Motility”, with software version 12.1c (settings were as advised by HT in the manual, chamber depth: 20 µm, temperature: 37°C).

Height measurement

The heights of 64 slides (128 chambers) of each brand were determined in the center using interferometric measurements with a specially adopted spectrophotometer and software. By comparison of the interferences of reflected and emitted light of the visible light spectrum the chamber height is calculated. The precision of this method is 0.2 µm.

Influence of the chamber on motility parameters

Motility assessments throughout the chambers were done with processed human sperm ($100 \times 10^6/\text{ml}$) and with boar sperm ($65 \times 10^6/\text{ml}$) using 4 chambers per sample. Boar sperm was used for its high sensitivity for toxic components from ink, glue, and coatings. Motility and progressive motility were determined in the center of the chamber (average of 4 different fields) and at the edges (average of 4 different fields at different edges), at 1, 5, 15, and 30 minutes (human) and 1, 3, 6, 10, and 20 minutes (boar). Results from different slides were averaged and the SD was calculated.

Filling behaviour

16 slides (32 chambers) of each brand were filled with processed sperm and an equal number with a liquefied ejaculate. During and after filling various aspects were evaluated: filling volume, shape of the loading front, smoothness of filling, and the number of air bubbles in the chamber after filling. As with the naked eye hardly any air bubbles were visible, the number was counted using the microscope.

Accuracy of concentration and motility determination

1. Disposable chambers/CASA

Two different processed sperm samples (32 chambers/sample) and three different liquefied ejaculates (12 chambers/sample) were used for the determination of concentration, and motility values. Random fields were selected manually (in an area about 2-3 mm from the edges) for CASA. A total of 250 cells (± 50) was analyzed per chamber. Mean value, minimum/maximum, and standard deviation (SD) per sample and per chamber type were calculated.

2. Flow cytometry (FACS)

The two processed samples and two liquefied ejaculates were diluted (10, 20, or 25x, depending on the initial concentration) in saturated NaHCO_3 ; dilution was performed with calibrated pipettes 10 separate times, and 100 µl of each dilution was pooled.

Four times, 50 µl of these pooled suspensions were brought into 900 µl of DNA staining solution (LDS-751 Quinaldin in PBS/0.1% BSA) together with 50 µl of beads suspension (1026/µl, Flow-Count Fluorospheres, Beckman Coulter).

20.000 events were analyzed by the FACS (Calibur, Becton Dickinson). Additional information regarding the gating of the FACS results is in progress and will be added in a later

stage. The signal of LDS positive events (R2) was related to the beads signal (R1), and the formula $(R2/R1) \times 1026 \times \text{dilution}$ gives the sperm concentration.

Sperm distribution

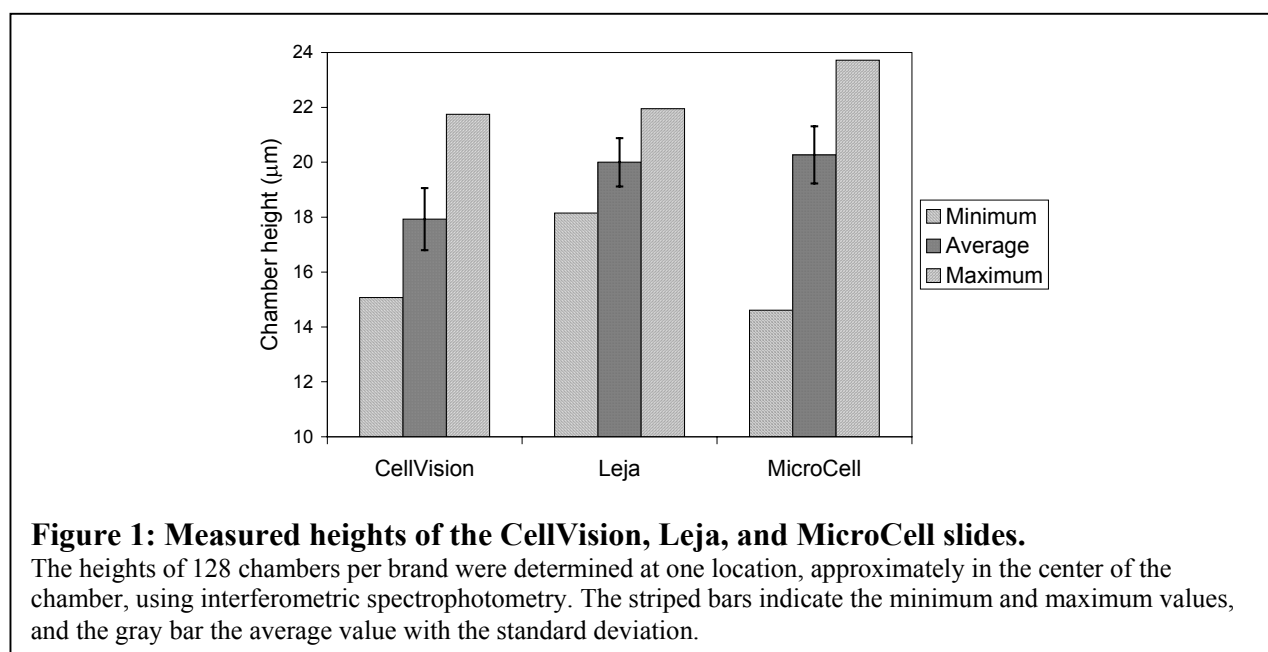
Sperm distribution throughout the chambers was evaluated for the Leja and MicroCell slides. At various positions in the chamber, the concentration was determined: for Leja at 6 positions along the horizontal middle axis and 8 along the vertical middle axis and for MicroCell, at 7 along the horizontal and 6 along the vertical middle axis. Per chamber the data were averaged, and the percentage deviation from this average was calculated per position. Next, the deviations per position were averaged for 8 different chambers.

RESULTS AND DISCUSSION

Height measurements

The specifications of disposable slides indicate a chamber height of 20 μm . Obviously, deviations from this height result in aberrant concentration determination. Using interferometric spectrometry, we measured the heights of 128 chambers per slide brand, and the results are shown in Figure 1.

The average height of the Leja chamber equals 20.0 μm , whereas the MicroCell is slightly higher (20.3 μm). The CellVision is more than 2 μm lower, and displays a large range of 6.7 μm . The range of the MicroCell seems larger (9.1 μm), but it has to be mentioned that the minimum value of 14.6 μm is an outlier. The next value is 18.6 μm , resulting in a range of 5.1 μm , and a SD of 0.91. The range and SD of the Leja slides are the smallest: respectively 3.8 μm and 0.88, indicating that the Leja SCAC has a height closest to 20 μm .



Filling behaviour of the slides

Easy filling is one of the advantages of disposable slides; it is fast and no complex protocols or explanations are needed. To check whether these three slide brands indeed display this convenience, 32 chambers were filled with processed sperm and 32 chambers with a liquefied

ejaculate. Various aspects were analyzed during and after filling and the results are shown in Table 1.

Two significant observations were made. First, the MicroCell chamber fills with an irregular shaped front, resulting in small air bubbles in the chamber. These bubbles can interfere with the counting process using CASA as well as during randomly choosing images by a lab technician. In the Leja chambers, air bubbles are hardly present after loading processed sperm; the angle of contact is probably lower than in the MicroCell slide, due to different surface properties of the glass. More bubbles become visible in the Leja chambers with the liquefied ejaculate, but not as many as in the MicroCell chamber. The CellVision chambers fill much slower than the other two, and this probably results in no air bubbles in the chambers with processed sperm (the experiment with the ejaculate was not performed; the reason will be explained later).

Second, the filling volumes indicated on the slides (5 µl for the Leja and 3 µl for the MicroCell) are tight. On average the Leja SCAC needs 5.3 µl and the MicroCell chamber 3.4

Table 1: Filling properties of the CellVision, Leja, and MicroCell slides.

32 chambers per brand were filled with processed sperm and 32 with a liquefied ejaculate. During and after filling, aspects mentioned in the Table were analyzed. With these sperm samples every chamber filled completely.

* Filling was very slow, ND is not determined.

<u>Processed sperm</u>	CellVision	Leja	MicroCell
Shape of the loading front:			
Slightly curved	32	32	1
Irregular			31
Chamber fills:			
Smoothly	32*	32	32
Jerkily			
Filling volume:	3.2 µl	5.3 µl	3.4 µl
Air bubbles after filling:			
0	32	25	1
1-3		7	2
3-9			6
≥ 10			23
<u>Liquefied ejaculate</u>	CellVision	Leja	MicroCell
Shape of the loading front:			
Slightly curved	ND	20	0
Irregular		12	32
Chamber fills:			
Smoothly	ND	31	32
Jerkily		1	
Filling volume:	ND	5.3 µl	3.4 µl
Air bubbles after filling:	ND		
0		3	0
1-3		19	0
3-9		9	12
≥ 10		1	20

µl.

Potential toxicity of the slides, as measured by assessing motility parameters

An often-raised disadvantage of disposable slides is toxicity from ink, glue, or coating. This effect results in loss of motility of sperm cells, and therefore, an underestimation of motility

parameters. We tested the time period that the sperm cells retain full motility at 37°C within the three types of slides. This experiment was performed with human as well as with boar sperm. Boar sperm is much more sensitive for toxic components, and therefore allows a more precise view on the toxicological effect of slides. Sperm was loaded into the chambers and motility measurements were done at various time points in the center of the chamber, as well as near the edges, where the concentration of toxic components (from glue and ink) is supposed to be highest.

The results with human sperm are shown in Figure 2. It is immediately apparent that motility of sperm cells is highly influenced by components of the CellVision slide. Already after 1 minute, both motility values are far below the levels as measured using the other two slides. We therefore conclude that the CellVision slide cannot be used for determination of motility. For this reason we omitted some experiments with this slide.

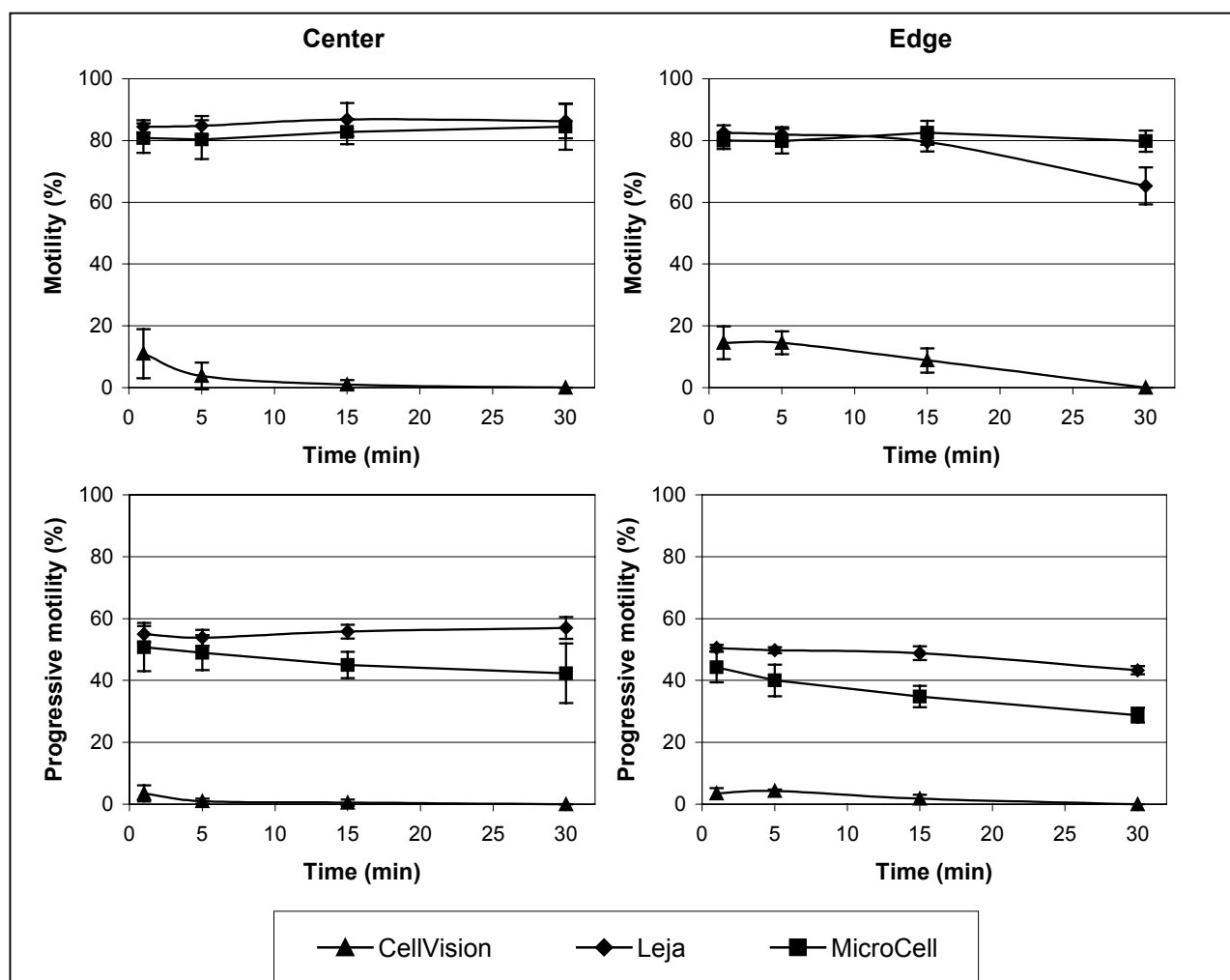


Figure 2: Motility measurements of human sperm in the CellVision, Leja, and MicroCell slides.

Motility and progressive motility of a processed human sperm sample were determined at 1, 5, 15, and 30 minutes after loading, in the center of the chamber and near the edges. Percentage motility is plotted against time.

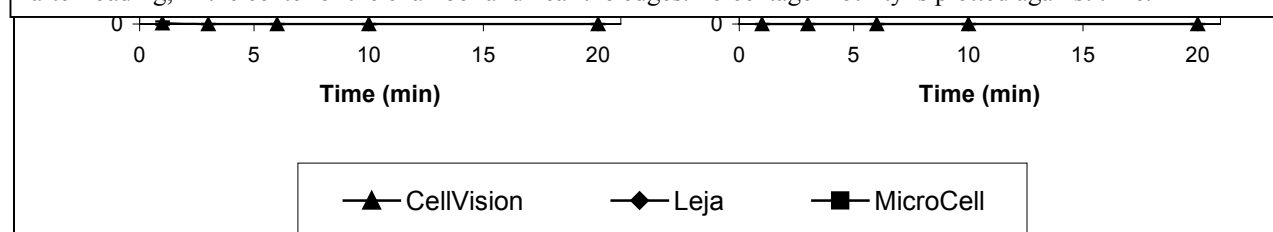


Figure 3: Motility measurements of boar sperm in the CellVision, Leja, and MicroCell slides.

Motility and progressive motility of boar sperm were determined at 1, 3, 6, 10, and 20 minutes after loading, in the center of the chamber and near the edges. Percentage motility is plotted against time.

In both the Leja and MicroCell slides the high motility levels remain constant, in the center for at least 30 minutes. Near the edges motility in the Leja slide is slightly decreasing between 15 and 30 minutes, and progressive motility is slowly decreasing in both slides, indicating that after about 15 minutes some negative influence is exerted by the edges. Under normal operating conditions, analysis of a sperm sample is complete within 5 minutes, therefore both the Leja and MicroCell slides can very well be used for motility measurements.

Between 1 and 10 minutes, motility values within the MicroCell slide are slightly lower than in the Leja slide. This phenomenon was more clearly observed in a similar experiment with boar sperm (Figure 3). Both motility and progressive motility of boar sperm in the MicroCell slide are considerably lower as compared to the Leja slide, and this reduction is most prominent near the edges. Furthermore, motility measurements show a larger deviation using the MicroCell slide. No extensive research was initiated to investigate these phenomena, but an interesting observation was made. Motility of boar sperm is not constant throughout the MicroCell chamber: for example, from entry to the end of the chamber motility/progressive motility gradually changes from 85%/50% to 25%/7%, which can explain the large SDs (this was not observed for human sperm).

Similar as for human sperm, the motility levels do not significantly decrease within 20 minutes, and therefore a direct toxic effect is not a likely explanation for the lower motility levels in the MicroCell slide. The cause could be mechanical. For example: the coating, or pressure induced by a higher evaporation at the air vent reduces motility of boar sperm cells.

Concluding, the CellVision slide cannot be used to determine motility of sperm cells. The Leja and MicroCell slides are non-toxic within operating time and can very well be used for motility measurements of human sperm, although the MicroCell gives a small underestimation. Only the Leja slide can be used for boar sperm.

Accuracy and reliability of concentration and motility determination

An important purpose of disposable counting slides is precise and reliable determination of the concentration of a sperm cell sample. Counting two processed and three liquefied semen samples in a large number of slides investigated this aspect. All results are summarized in Appendix 1 and 2. For visualization, the results of processed sample 1 and ejaculate 1 are graphically depicted in Figure 4.

For processed samples, the concentrations as measured in the Leja and MicroCell slides are very close to each other, and also to the FACS outcome. Also for the ejaculates, counting in the Leja and MicroCell slides results in very similar concentrations. The FACS outcomes however are much higher. This is probably caused by bacteria present in the ejaculate, which are stained by LDS-Quinaldin, and are counted as sperm cells. In the concentration measurements, all SDs, and the ranges except for one, are smaller for the Leja chamber than for the MicroCell.

The results with the CellVision slides are difficult to interpret. As a result of the high percentage of non-motile cells, the HT CASA-system has difficulties identifying the sperm cells. To overcome this problem, gating of various parameters was adjusted. Depending on the quality of the sample, debris can then be included in the measurement, resulting in an overestimation, or sperm cells are still omitted, resulting in an underestimation. Motility and progressive motility are too low in the CellVision chamber, as was also shown in Figures 2 and 3. This effect is more pronounced with processed samples than with the ejaculate; semen fluid could partially protect the sperm cells from the chamber toxicity.

As observed and discussed earlier, motilities as measured in the MicroCell chamber are a few percent lower than the values in the Leja chamber. The SDs and ranges for both chambers are comparable (appendix 1 and 2).

Concluding, both the MicroCell and the Leja slides are precise and reliable counting chambers for sperm cells. However, the Leja slide gives the highest motility values, and the smallest

concentration deviation, and therefore has a higher probability of giving a value very close to the real concentration.

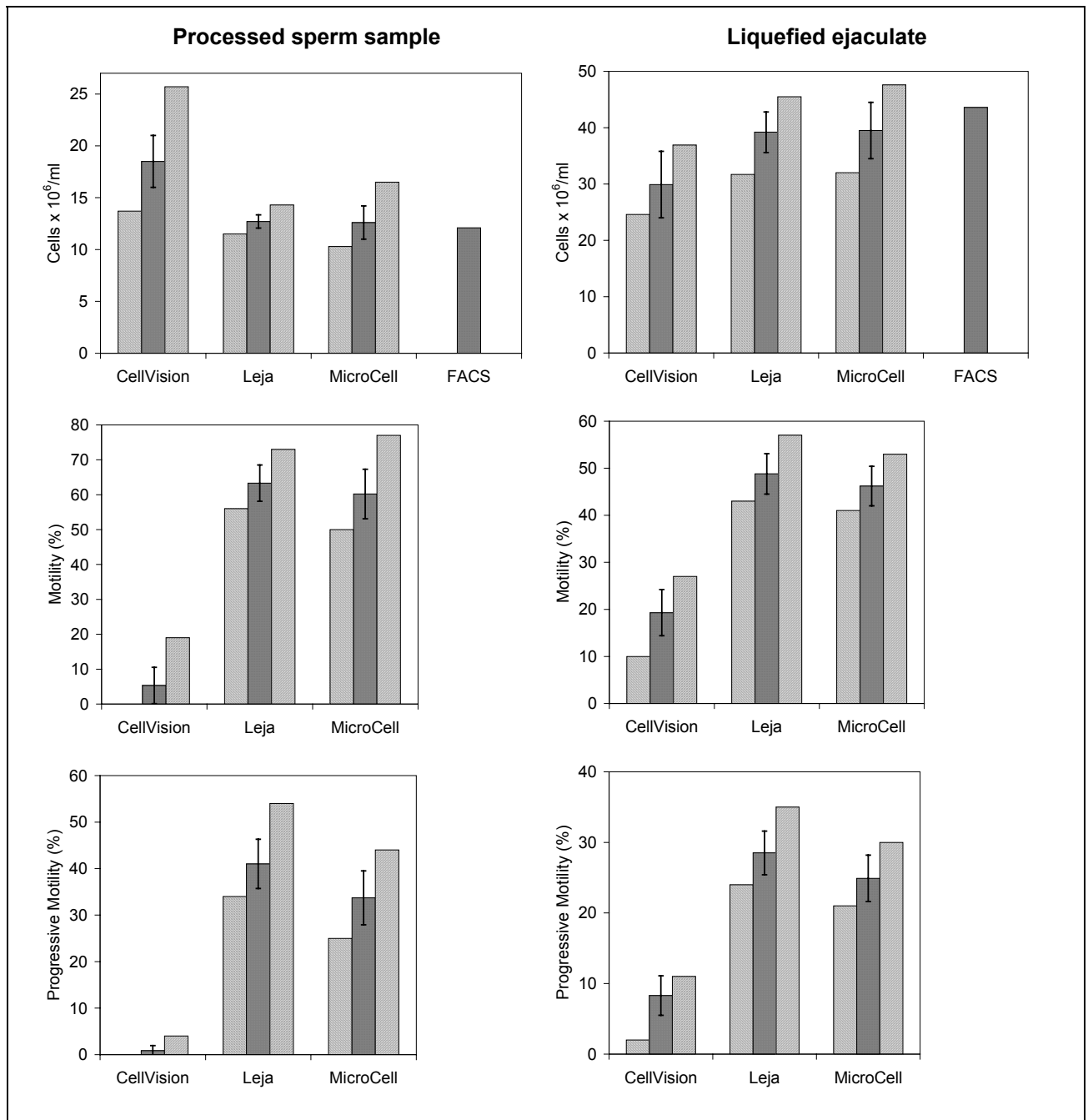


Figure 4: Concentration determination and motility measurements of human sperm using the CellVision, Leja, and MicroCell slides.

The concentration and motility data from processed sample 1 and ejaculate 1 (as presented in Appendix 1 and 2), are graphically shown here. As well as for concentration and motility, the striped bars indicate the minimum and maximum values, and the gray bar represents the average value (from respectively 32 and 12 data) with the standard deviation. No SD for the FACS value is given. The value presented here is the average of 4 different dilutions. The belonging SD would indicate the accuracy of diluting, and not of the FACS method. When the same dilution is analyzed twice by the FACS, the same value is obtained.

Sperm cell distribution throughout the chambers

A discrepancy between the results with the Leja and MicroCell slides arose during this investigation. The SDs belonging to the height measurements are comparable, whereas the SDs and ranges of the concentration measurements for the Leja slides are smaller than for the MicroCell slides.

To find an explanation for this, we studied the intrachamber variation within the Leja and MicroCell slides. At various positions in the chambers the concentrations were determined and the deviation per position was calculated (Table 2). For the Leja slide, the deviations at all 14 positions are smaller than 10%, indicating that the sperm cells evenly distribute through the chamber. In the MicroCell slide two outliers are detected: +70.3% at the end of the chamber, and -18.5% at the entry.

Concentration measurement can easily be influenced when locations with high deviations are selected by the CASA system or are chosen by the lab technician. Including a position at the end of the chamber results in an overestimation, whereas when positions in the vicinity of the end are omitted, the result is an underestimation.

Concluding, the homogeneous distribution of sperm cells in the Leja chamber probably results in the small range and SD, and therefore in a more reliable concentration determination.

Table 2: Distribution of sperm cells throughout the Leja and MicroCell chambers.

In 8 chambers, concentrations were determined at various positions within the chambers (14 for the Leja and 13 for the MicroCell chamber). Per position, the deviations from the average value were determined and the deviations per position were averaged. The highest deviations are underlined.

	Leja	MicroCell									
<i>Top</i>	-3.9										
	4.0	3.4									
	1.3	-9.2			<i>Entry side</i>				<i>End of chamber</i>		
	-6.0	-5.8	Leja	1.0	-2.8	4.0	3.4	2.9	6.7		
	-7.4	-2.8	MicroCell	<u>-18.5</u>	0.0	-1.9	-0.4	-8.5	-10.7	<u>70.3</u>	
	4.2	-5.8									
	-0.8	-10.1									
<i>Bottom</i>	-7.1										

ABBREVIATIONS

CASA	Computer-assisted semen analysis
FACS	Fluorescence activated cell sorter
HT	Hamilton Thorne
HTF/HSA	Human tuba fluid/human serum albumin
ND	Not determined
SCAC	Standard Count Analysis Chamber
SD	Standard deviation

Appendix 1: Concentration determination and motility measurements of human processed sperm.

Two different human processed sperm samples were used for concentration determinations in 32 CellVision, Leja, and MicroCell chambers. The concentrations were also determined using the FACS. During concentration measurements using CASA (HT), the motility parameters were determined simultaneously. Mean (progressive) motilities were calculated over the first 20 measurements for sample 1 and over the first 10 measurements for sample 2, as thereafter the quality of the samples rapidly decreased.

* Concentration in 10^6 cells/ml. ** (Progressive) motility in %.

<u>Sample no. 1</u>		CellVision	Leja	MicroCell	FACS
Concentration*	mean	18.5	12.7	12.6	12.1
	SD	2.5	0.64	1.6	
	min / max	13.7 / 25.7	11.5 / 14.3	10.3 / 16.5	
	range	12	2.8	6.2	
Motility**	mean	5.3	63.3	60.2	
	SD	5.2	5.2	7.1	
	min / max	0 / 19	56 / 73	50 / 77	
	range	19	17	27	
Prog. motility**	mean	0.84	41.0	33.7	
	SD	1.1	5.3	5.8	
	min / max	0 / 4	34 / 54	25 / 44	
	range	4	20	19	
<u>Sample no. 2</u>		CellVision	Leja	MicroCell	FACS
Concentration*	mean	40.2	45.3	43.7	43.5
	SD	5.9	3.1	5.7	
	min / max	29.2 / 50.4	38.7 / 50.2	33.3 / 54.9	
	range	21.2	11.5	21.6	
Motility**	mean	4.6	74.2	63.7	
	SD	6.4	5.8	7.4	
	min / max	0 / 28	64 / 83	55 / 73	
	range	28	19	18	
Prog. motility**	mean	1.3	49.9	46.6	
	SD	2.1	4.1	6.1	
	min / max	0 / 9	41 / 54	38 / 55	
	range	9	13	17	

Appendix 2: Concentration determination and motility measurements of human liquefied ejaculates.

Three different human liquefied ejaculates were used for concentration determinations in 12 Leja and MicroCell chambers, and one sample was counted in 12 CellVision chambers. Two concentrations were also determined using the FACS. During concentration measurements using CASA (HT), the motility parameters were determined simultaneously. * Concentration in 10^6 cells/ml. ** (Progressive) motility in %.

<u>Sample no. 1</u>		CellVision	Leja	MicroCell	FACS
Concentration*	mean	29.9	39.2	39.5	43.6
	SD	5.9	3.6	5.0	
	min / max	24.6 / 36.9	31.7 / 45.5	32.0 / 47.6	
	range	12.3	13.8	15.6	
Motility**	mean	19.3	48.8	46.2	
	SD	4.9	4.3	4.2	
	min / max	10 / 27	43 / 57	41 / 53	
	range	17	14	12	
Prog. motility**	mean	8.3	28.5	24.9	
	SD	2.8	3.1	3.3	
	min / max	2 / 11	24 / 35	21 / 30	
	range	9	11	9	
<u>Sample no. 2</u>		CellVision	Leja	MicroCell	FACS
Concentration*	mean	ND	64.6	65.8	97.0
	SD		4.1	4.7	
	min / max		56.8 / 72.0	58.7 / 73.4	
	range		15.2	14.7	
Motility**	mean	ND	33.8	34.8	
	SD		3.9	4.4	
	min / max		30 / 40	27 / 41	
	range		10	14	
Prog. motility**	mean	ND	20.7	18.2	
	SD		2.3	3.3	
	min / max		16 / 24	13 / 23	
	range		8	10	
<u>Sample no. 3</u>		CellVision	Leja	MicroCell	FACS
Concentration*	mean	ND	38.1	37.8	ND
	SD		3.0	3.9	
	min / max		34.0 / 44.9	30.8 / 44.2	
	range		10.9	13.4	
Motility**	mean	ND	41.1	38.9	
	SD		3.0	2.4	
	min / max		35 / 45	34 / 43	
	range		10	9	
Prog. motility**	mean	ND	33.0	30.9	
	SD		3.3	2.8	
	min / max		27 / 38	25 / 35	
	range		11	10	