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# Simple optical method of qualitative assessment of sperm motility: preliminary results

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## ABSTRACT

The examination of quality of the sperm ejaculate is one of the most important steps in artificial fertilization procedure. The main aim of semen storage centres is to characterise the best semen quality for fertilization. Reliable information about sperm motility is also one the most important parameters for *in vitro* laboratory procedures. There exist very expensive automated methods for semen analysis but they are unachievable for most of laboratories and semen storage centres.

Motivation for this study is to elaborate a simple, cheap, objective and repeatable method for semen motility assessment. The method enables to detect even small changes in motility introduced by medical, physical or chemical factors. To test the reliability of the method we used cryopreserved bull semen from Łowicz Semen Storage Centre. The examined sperm specimen was warmed in water bath and then centrifuged. The best semen was collected by the swim-up technique and diluted to a proper concentration. Several semen concentrations and dilutions were tested in order to find the best probe parameters giving repeatable results.

For semen visualization we used the phase-contrast microscope with a CCD camera. A PC computer was used to acquire and to analyse the data. The microscope table equipped with a microscope glass pool 0.7mm deep instead of some conventional plane microscope slides was stabilised at the temperature of 37°C.

The main idea of our method is based on a numerical processing of the optical contrast of the sperm images which illustrates the dynamics of the sperm cells movement and on appropriate analysis of a grey scale level of the superimposed images. An elaborated numerical algorithm allows us to find the relative amount of motile sperm cells. The proposed method of sperm motility assessment seems to be objective and repeatable.

**Key words**: bull semen, *in vitro* fertilization, phase-contrast microscope method, optical imaging, semen motility parameters

## 1. INTRODUCTION

In humans, as in animal species, the relationship between semen characteristics and *in vivo* or *in vitro* fertility outcome is not very clear yet. Motility is commonly believed to be one of the most important characteristics associated with the fertilizing sperm ability. Indeed, motility has been recognized for a long time as essential for the transport of sperm cells through the female reproductive tract and for fertilization. Motility is an expression of viability and structural integrity of the spermatozoa. Morphological parameters of spermatozoa are expressed also in motility and are directly correlated with physiological events during fertilization and logically with reproduction outcome. Especially in *in vitro* procedure where for good results the best quality of sperm ejaculate is need, evaluation of sperm vitality is of special importance.

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In many laboratories the sperm mobility assessment is made with use of a conventional microscope observation by trained personnel according to rather subjective criteria due to the individual skill of a person performing the analysis. During such estimation of concentration and of mobility of sperm cells the important errors can be introduced. In humans and animals variations of 30 to 60% have been reported in estimated motility parameters of the same ejaculates (1, 2, 3, 4, 5, and 6). In particular the subjectivity of the analysis makes any comparison of results difficult or impossible.

To overcome this ambiguousness, different systems have been proposed such as turbidimetry (7), laser-Doppler spectroscopy (8), computer-assisted sperm analyzers (9, 10, 11) and photometric methods (12). However, because of high price and degree of complexity such instrumentation is not accessible for many laboratories and for most semen storage centers. In addition, such systems need special, sometimes expensive equipment, very good trained personnel often with a background in physics.

The purpose of this study was to find some simple, cheap, objective and repeatable method for the semen motility assessment which can be used in a common storage centre as well as for our further experimental trials. We have proposed the method of the processing of the optical contrast of the sperm images illustrating dynamics of the sperm cells movement and the appropriate analysis of a grey scale level of the superimposed images. The elaborated numerical algorithm gives us information about the relative sperm motility.

The presented method of sperm motility assessment is a process that involves three successive steps. The first one concerns the sample preparation (washing, dilution, centrifugation, etc.); the second one concern the image acquisition with use of the negative phase-contrast microscope connected to the CCD camera; and the last one is about the image acquisition and the processing method.

Specimen staining, microscope magnification and system optics have been chosen to optimise the properties of the data stored by a PC computer coupled via the fire-wire connection to the camera. Those parameters are essential and are known to be able to change significantly the results of measurements.

## 2. MATERIALS AND METHODS

### 2.1. Semen Collection and preparation

The semen was obtained from ten bulls from Łowicz Storage Center in some frozen straws. They were stored in liquid nitrogen temperature and thaw in temperature of 37°C just before the experiment.

The sperm cell concentration for each straw was assessed by the storage center before a frizzing procedure. A low sperm concentration was defined as $<10 \times 10^6$ sperm/mL, a medium concentration as between $20 - 40 \times 10^6$ sperm/mL, and a high concentration as $> 150 \times 10^6$ sperm/mL. Sperm counts were performed with use of a hemacytometer method.

For a swim-up procedure a 1:20 dilution of semen was prepared with PBS (Phosphate Buffer Saline) and BSA (Bovine Serum Albumine).

### 2.2. Swim-Up Procedure

Intact ejaculated spermatozoa may be damaged by oxygen radical emanating from seminal leukocytes and damaged spermatozoa (13, 14), so that the stress caused by the cryopreservation procedure itself may add to damage inflicted already by free oxygen radicals (15). It is not known if the processes of freezing and thawing damage all spermatozoa equally, or if there is selective damage to a more susceptible population such as that suffering of lipid peroxidation.

The swim-up procedure eliminates the seminal plasma and immotile and dead spermatozoa along with exfoliated epithelial cells, cellular debris, leukocytes and amorphous material. Spermatozoa selected by the swim-up method possess better characteristic in the sperm penetration assay than the untreated specimens (16). In this procedure we select the most mobile spermatozoa from all ejaculate.

An aliquot of washed semen was centrifuged at 208x*g* for 10 minutes, and the supernatant was discarded. The sperm pellet was overlaid gently with 0.5 mL of STL, followed by 30 minutes incubation at the temperature of 37°C in water bath to allow the motile spermatozoa to swim up into the upper layer of the medium, which was then separated. The semen prepared in that way was than diluted to the proper concentrations and placed at the temperature of 37°C in water bath for incubation.

### 2.3. Effects of Temperature

It is known, that temperature strongly influences the motility of spermatozoa. Many studies have been performed in different species in order to determine the optimal temperature, which appeared to be the temperature closest to the body normal temperature. The temperature of 37°C seems to be recommended in humans and bulls, whereas 38°C is often the temperature of choice in other animal studies. In dogs, Iguer-Ouada and Verstegen (17) analyzed the effects of two temperatures: 30°C which corresponds to the semen temperature after ejaculation, and 38°C which is the canine body temperature. The conclusions were that semen motility parameters were clearly influenced by the temperature at assessment, and all studied parameters were decreased at 30°C. In particular analysis of semen at room temperature which is usually lower than the body temperature and in general is not constant, can significantly affect results of examination. That's why our studies where performed at the temperature of 37°C which was the optimal temperature for bull sperm cells under examination. Therefore a heated microscopic table and a home-made sperm incubator were prepared for these studies with temperature stabilization at 37°C ± 0.5°C.

### 2.4. Effects of Sperm Concentration

A strong influence of sperm cells concentration on results of optical investigations has been noticed. Semen must be diluted before analysis because the sperm cell concentrations of raw semen are too high for the analysis of individual spermatozoa tracks. The extender used to dilute semen must not contain particles of a size similar to sperm cells heads (e.g., non-clarified egg yolk and whole milk extenders), because they would not be distinguished from non-motile sperm.

The sperm concentration has to be carefully chosen by a proper sperm dilution. The aim is the optimization of a number of sperm cells for a proper semen imaging within the visual field of the microscope and for microscope optical depth corresponding to the proper magnification chosen for the study of semen spatial dynamics.

### 2.5. Data Acquisition

For sperm visualization dynamics we used the negative phase contrast microscope (PZO) integrated with a CCD camera connected to a PC computer via a FireWire connection. The dynamics of the sperm cell movement was registered by CCD camera as a series of images formed at the phase contrast microscope image plane. The resulting movies in .avi format have been analyzed frame by frame. The images acquisition frequency was an important parameter for the data analysis, as the resolution of reconstructed sperm trajectories is influenced by the frame rate (18, 19, 20) similarly as some others kinetic parameters (21, 22, 23). On the base of results from CASA (Computer Assisted Semen Analysis) experiments with humans, it is currently accepted that sperm motion in capacitating medium should be analyzed at 50 to 60 Hz or more (24, 25) instead of 30 Hz. However, the choice of the frame rate has to be related to the particular specimen as well as to the experimental condition, detection and method of data analysis.

In our experimental trial we have found that the frequency of 50 or 30 Hz is too high for the bull sperm cells under study. We have found 9 frames per second as the optimal frame rate for our experiment. The number of frames taken to analyze was 50.

In general, more variations in results are observed for the analysis of different fields of view than for the repeated analysis of the same view field, so the largest the number of cells analyzed, the more reduced the coefficient of variation. Consequently, the precision of the results increases as the number of the view fields and analyzed cells increase. In CASA (Computer Assisted Semen Analysis) method according to bull semen the measure of 30 fields of view and approximately 300 cells is recommended (26, 37) whereas in the stallion from 300 to 500 cells seems to be optimal (28, 29). In our method we analyzed 5 fields of view for each sample. It was enough because the results were similar to each other with deviation of 1.5 %.

## 3. RESULTS

### 3.1. Sperm motility assessment.

Sperm motility was registered for each sample for diluted and non diluted spermatozoa at the beginning of the experiment (zero starting time), and repeated after the same periods of time: 30 min, 1 h, 1.5 h. During this process the samples were incubated in temperature of 37°C in the water bath.

Every movie illustrating sperm dynamics was analyzed frame by frame. As a first step of data processing we choose few areas lying within some single sperm cells (see Fig. 1) and we measure the average value of the gray scale level of those areas and we treat it as a thresholds value. Next we substitute the pixel gray level below the threshold by 0 (100% dark); this area is equivalent to the background and the pixel gray level above the threshold by 255 (100% white). After numerical processing of the entire frames movie we can see spermatozoa as some white spots (see Fig. 1) with high contrast on a black background. The whiteout area corresponding to sperm cells are displayed with much better contrast than at the original images from the phase contrast microscope (see Fig.1).

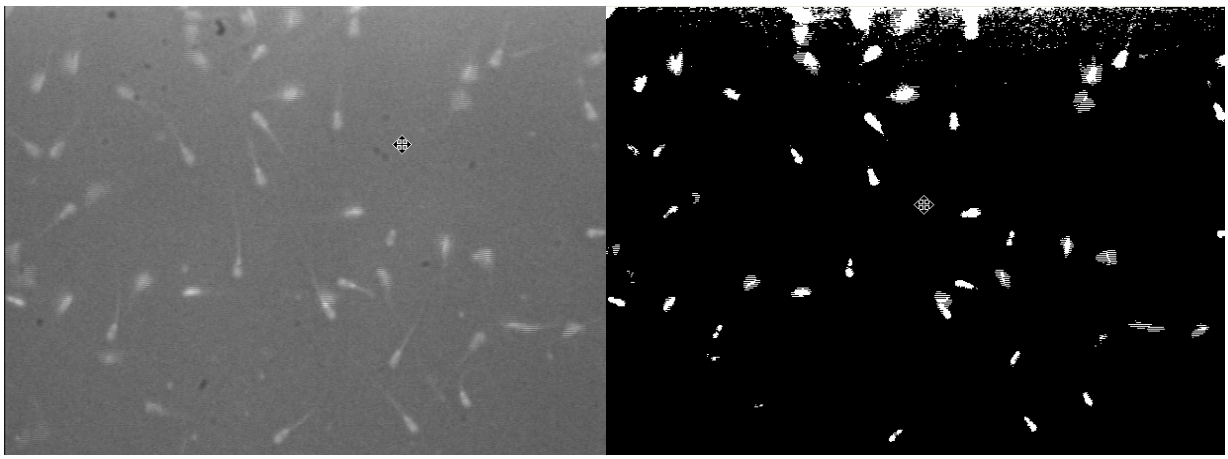

Figure 1: Comparison of movie fames before numerical processing (left) and after assigning the threshold value correspondingly (right).

In our experiment we found out that overlaying (superimposing) 50 frames in the analysis is enough to estimate the percent of mobility of tested sperm cells.

The image of the first frame is subtracted from the superposition in order to eliminate the immobile image fragments (dead sperm cells, unavoidable impurities of the sample and of the optical path) from the spontaneously moving ones.

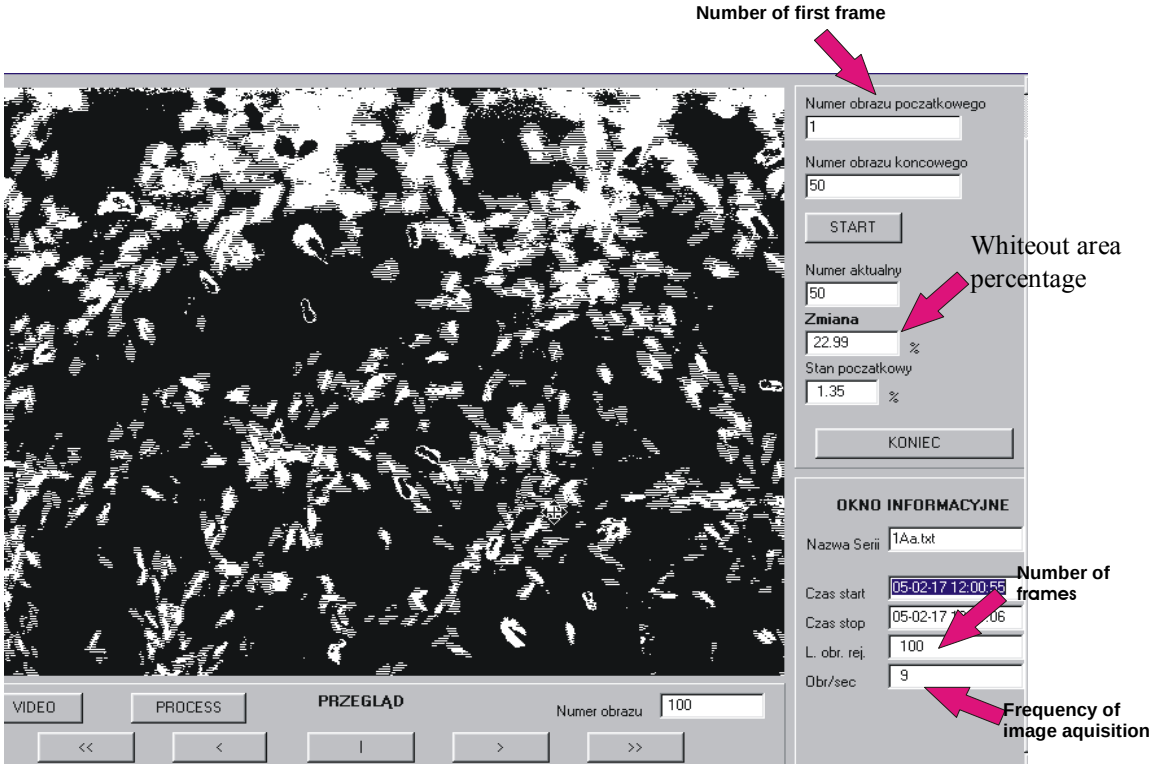


Figure 2: A working window of the analysing programs in use.

Evaluation of the gray scale level for the total area of the superimposed frames in comparison with the first one gives us the information about motility of the sperm cells in percents. For immobile cells the measured change in the total gray scale level would be 0%. The output percentage of the total gray scale level gives us the relative motility of the sperm cells allowing for studying the changes of the sperm vitality due to some external factors with precision corresponding to the accuracy of the measurement (less than 1%). Fig. 2 presents a program window illustrating some sample data after numerical processing.

### 3.2. Sperm motility estimation

The percentage of whiteout area of the final image corresponding to the superimposed frames after numerical processing described above gives us information about the relative amount of motile sperm cells.

To describe quantitatively the sperm motility $M$ we use the following relation:

$$M = c\,W,$$

where $W$ is the whiteout area percentage and $c$ is proportionality constant.

In general $c$ will depend on the type of species, sperm concentration, the frame rate and the time of acquisition. In our study we choose the value of $c$ basing on a kind of statistics of our measurement. Since our statistics is not very rich, we use the Empirical Rule for data sets: that is a normal, bell-shaped distribution with approximately 95% of all the data falling in the range of errors bellow the standard deviation ($2\sigma$) of the mean ($\langle x \rangle$). We assume for the value of $c$:

$$c = \frac{95\%}{W_{\max}},$$

where $W_{max}$ is the maximum value of whiteout percentage found in our experiment. Such a choice of $c$ value allows as introducing a common scaling for our motility measurements. We find our assumptions are correct because the deviation from the mean value is small for every experimental trial.

Now we can calculate the motility for each sample. For our investigation we have used 10 samples which were prepared and analyzed in the same conditions (time and temperature of incubation, frame rate). Below we present the results (Table I).

| **Sample number** | **W [%]** | **M [%]** | **$M_{30min}$ [%]** | **$M_{1h}$ [%]** | **$M_{1.5h}$ [%]** |
|---|---|---|---|---|---|
| 1 | 26 | 94 | 94 | 85 | 69 |
| 2 | 15 | 54 | 52 | 48 | 32 |
| 3 | 21 | 76 | 75 | 65 | 48 |
| 4 | 24 | 88 | 88 | 78 | 56 |
| 5 | 15 | 53 | 50 | 43 | 30 |
| 6 | 16 | 57 | 54 | 45 | 33 |
| 7 | 27 | 96 | 96 | 87 | 72 |
| 8 | 23 | 83 | 80 | 70 | 64 |
| 9 | 19 | 68 | 66 | 56 | 43 |
| 10 | 17 | 61 | 58 | 46 | 35 |

**Table I.** The calculation of sperm motility for ten samples with proposed procedure for several times of incubation; $W_{max} = 26.66$; $c = 3.6$; $\langle x \rangle = 20.34$; $\sigma = 4.3$.

The calculated percent of motility is in accordance with the qualitative estimate by the conventional microscopic evaluation obtained for each sample. Samples 1, 3, 4, 7, 8 were described as good quality with high motile sperm; Samples 2,5,6,9 and 10 were described as medium motile sperm. Our analysis confirmed those statements in a quantitative way.

From the table above we can notice that sperm motility drops significantly with time of incubation.

### 3.3. Sperm tracks observation.

The method presented in this study can be applied to the sperm tracks detection. Sperm trajectory could give us interesting information about the state of spermatozoa and its morphological condition. By choosing the proper frame rate of acquisition and the proper number of frames to analysis we can observe the track of the single spermatozoa. The shape of trajectories gives us the possibility of differentiating between hyperactivated, non-hyperactivated and damaged spermatozoa. Sperm trajectories can be characterised as belonging to three categories: ‘ideal tracks’, ‘long tracks’ and ‘circling tracks’ (28, 29, and 30). In our studies we observed all kind of these tracks (see Fig. 3)

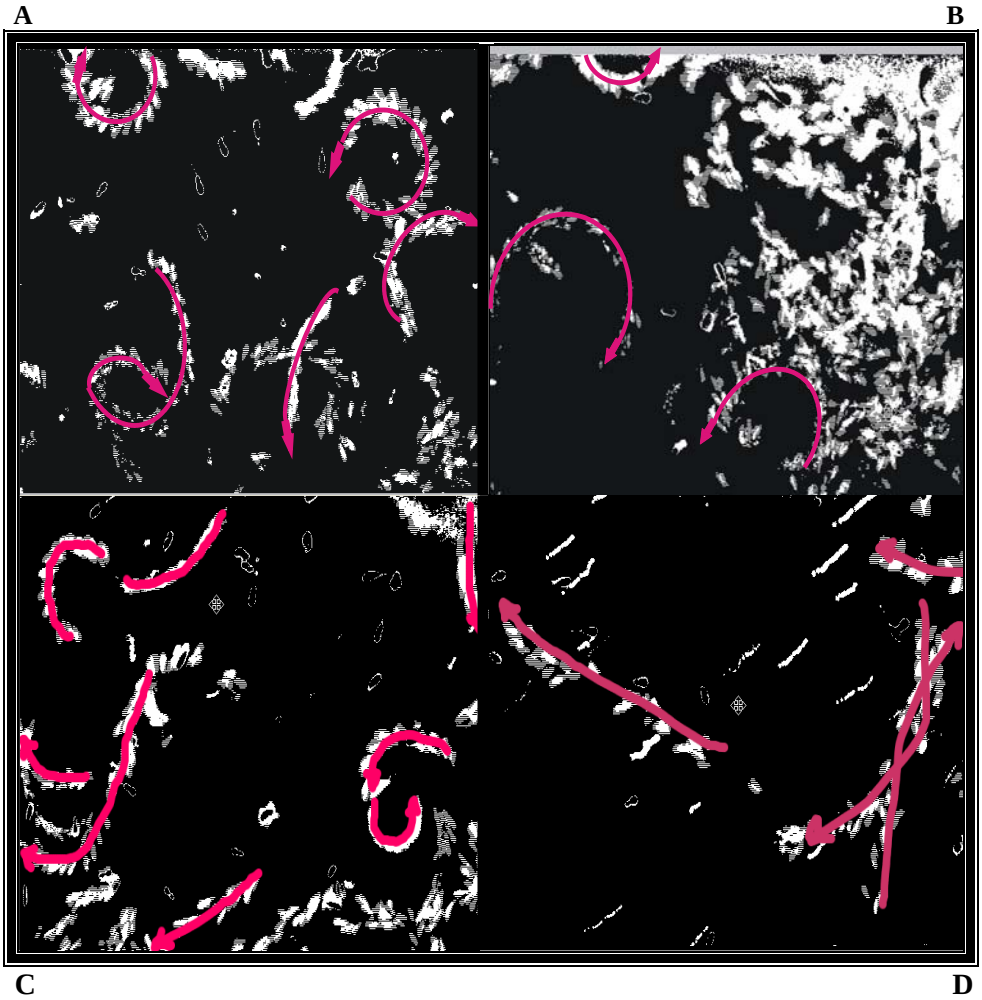

Figure 3: Comparison of kinematic profiles of spermatozoa (A, B – circling tracks corresponding to hyperactivated and damage sperm cells, C – long tracks, D- ideal tracks corresponding to non-hyperactivated sperm cells).

The information about the morphological conditions of semen is the most important in *in vitro* fertilisation procedure while the sperm morphology influences the final positive result.

## 4. DISCUSION

In the past and even now in most of storage centers, the conventional microscopic evaluation of sperm motility and concentration is considered the gold standard. However, because of its lack of objectivity and repeatability, other methods based on image analysis, photometry measurements, spectroscopy, micrography, have been tested. The most important goal in all these methods is to achieve the reliable information about semen quality. This goal seems to be also the main mission of semen storage centres, which should characterise the best semen for fertilisation. Reliable information about sperm motility, which determines the fertilisation conditions, is the most important for *in vitro* procedure. However, in the most of storage centres the conventional rough microscopic estimation of semen parameters is used. During routine laboratory examination trained personnel usually assesses the concentration and mobility of sperm cells, however due to subjectivity of the method grave errors are unavoidable. In particular that kind of analysis makes any comparison of results difficult or impossible.

In this study we present the simple method of sperm motility assessment which is based on numerically supported phase contrast technique of analysis of sperm images. Our study includes the choice of the proper optical

conditions of our experimental setup, as condenser rings alignment, proper magnification and choice of the proper frame rate, of the number of superimposed frames, and of the proper threshold for our numerical analysis procedure. Using statistics method we found the way of assigning the measured whiteout area due to the sperm cells track into a percentage of motile sperm cells.

In our investigation we used cryopreserved semen which after thaw never achieves the motility of fresh semen. The cryopreservation procedure is very sophisticated and stressing for cells therefore some amount of sperm cells die. As a result the motility of semen evaluated in our experiment is always a fraction of the motility of the most vital sperm. The introduction of the absolute scale for motility assessment is a complex problem depending on several parameters of the sperm and the detection of images and the applied numerical procedure. In further study we will try to solve that problem.

As a second outcome of the study, the velocity trajectories of hyperactivated and non-hyperactivated spermatozoa were found. In our experiment we observed that they look quite different (Fig. 3) and seem to be easy to recognize. The information about the amount of hyperactivated spermatozoa in ejaculate sample seems to be important for in vitro fertilization procedure.

In literature we can find that hyperactivated spermatozoa have a higher velocity than non-hyperactivated spermatozoa (29, 30). This subject will be investigated in further studies.

In conclusion we assert that the method introduced here provides some new simple solutions in analyzing sperm quality with results comparable to more expensive methods. It is obvious that more statistics should be done. We plan to continue further studies on sperm motility in different conditions.

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