



Deliverable Report

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Objective: The goal of this deliverable is to provide a tool for an objective comparison of sperm quality of male grey mullet subjected to different hormone-based treatments. The specific aim was to establish a Computer Assisted Sperm Analysis (CASA) for the evaluation of grey mullet sperm: The best adapted CASA parameters for grey mullet sperm analyses was determined and reported to end users to optimize their ability to check fertility potential of the semen in the course of their future spawning induction experiments.

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Introduction

In nature, the grey mullet (*Mugil cephalus*) spawns only in seawater, and the reproductive season of the Mediterranean population ranges from July to December (Abraham *et al.*, 1966). However, under captive conditions, grey mullet breeders do not spawn spontaneously, with both genders displaying reproductive dysfunctions, even in full strength seawater (Aizen *et al.*, 2005). In Hawaii, cultured grey mullet males have been found to mature and regress earlier than females (Weber and Lee, 1985), and even then, in most cases, the produced milt was highly viscous and failed to fertilize the eggs (Shehadeh *et al.*, 1973). A different situation has been found in the Mediterranean grey mullet brooders, where males with running milt are rarely observed (De Monbrison *et al.*, 1997; Yashouv, 1969). To augment testicular development, grey mullet males were treated early on in the reproductive season with methyl testosterone (MT) administered *via* Ethylene vinyl acetate (EVAc) slow-release implants. This was found to be a potent spermiating procedure, which enhanced milt production among treated males (Aizen *et al.*, 2005). Despite the reported success, further optimization of the hormonal treatment is still needed to be done. In this regard, once established, an accurate means for sperm quality evaluation, such as CASA, is expected to provide the basis for tailoring the optimal hormone-based therapy required to induce synchronized spermiation over prolonged period among grey mullet males.

Description

The establishment of the CASA protocol for analyzing the grey mullet sperm motility was launched at the onset of DIVERSIFY by technology transfer to be used at P4. IOLR facilities. A training course for Dr Iris Meiri Ashkenazi from this partner was programmed to be held in the experimental station of aquaculture P14. IFREMER at Palavas les flots, France. Initially, the training was planned on European seabass (*Dicentrarchus labrax*) sperm, a model species well studied by Dr. Christian Fauvel and colleagues. However, when the training course was finally possible (May, 2014), European seabass were already out of their reproductive season, and training was made with meagre (*Argyrosomus regius*) sperm provided by a non-partner private company, “Les Poissons du Soleil”, Balaruc, France).

The sperm samples from 6 males (belonging to the commercial broodstock) were either chilled stored or cryopreserved in the hatchery and brought back to P14. IFREMER laboratories for evaluation. Both types of storage were efficient, facilitating training sessions during which procedures were demonstrated, practiced and all doubts were discussed and solved immediately. A detailed generic protocol of sperm analysis was written and provided below entitled “Vademecum for the analysis of sperm motility: General protocol and propositions for grey mullet sperm quality assessment”, the objective of which is to provide a ready-to-use help for the implementation of the method.

The second step consisted of a first trial with grey mullet sperm in IOLR facilities including sperm preparation and video recording. The movies were sent to P14. IFREMER in order to adapt the CASA settings to the particular case of grey mullet. Mail exchanges allowed the group of Israeli and French partners to communally define a protocol for grey mullet sperm analysis and provide the preliminary results reported below later in this deliverable.

Vademecum for the analysis of sperm motility: General protocol and propositions for grey mullet sperm quality assessment

The production of domestic individuals relies on reproduction success and particularly on the success of fertilization. As a general feature, temperate zone fish breed seasonally after gametogenesis, the duration of



which varies according to the species. During mating of most fishes, gametes are released by both sexes in the water, where fertilization occurs so that both intrinsic gamete quality and quantity, and environment of gamete fusion determine fertilization success.

In species diversification for aquaculture, it is necessary to provide broodstock as well as gametes with adequate environmental and biological conditions. For this, the analytical assessment of sperm quality in various standardized micro-environmental conditions may allow a better adjustment of broodstock and gamete management. The current document does not pretend to be a scientific report, but it aims at establishing a reliable tool for sperm quality assessment through a free software developed by The National Institutes of Health of the United States, for which a CASA plugin was designed (Wilson Leedy and Aldermann, 2007).

1) General features of temperate marine fish male reproduction

- Discontinuous spermatogenesis (seasonal) and possible disconnection between spermatogenesis and spermiation (according to species)
- Male fluency covering the whole spawning period of the females
- Ageing of sperm (variation of quality along the reproductive season)
- Collection of sperm by simple pressure of fish belly (more or less easy according to species)

2) General features of fish sperm

Concentration/Volume of expressible milt

- Species specific: volume from only 10 μ l (e.g., Senegalese sole *Solea senegalensis*) to more than 20 ml; concentration from 1×10^9 to 6×10^{10} spermatozoa (szoa) ml^{-1}
- Both may decrease along the reproductive season

Motility

- No motility in genital tract
- Motility triggered by external medium at ejaculation.
- In marine fish motility is triggered by hyperosmotic shock (seawater as well as glucose)
- Motility is high at activation, then it decreases until exhaustion
- Motility is sensitive to environmental pH
- Motility duration is variable
 - Linked to intrinsic energy stores, since spermatozoa respiration does not compensate their ATP expenses due to motility.
 - Species specific from 30 sec in trout to 15 min in bluefin tuna (*Thunnus spp*)
 - Varies along the season (European seabass, turbot *Scophthalmus maximus*)

Sperm fertilization ability

- Depends on species (optimized artificial fertilization for 6×10^3 szoa/egg in turbot and 7×10^4 spz/egg in European seabass, for instance)
- Depends on time in the season
- More or less sensitive to storage, e.g., from several days in sturgeons *Acipenser spp* or trout *Oncorhynchus mykiss* to several hours in European seabass.

Video recording

A) Material

- 1) Install a videocamera on the microscope (in our case USB digital camera ref DMK 22BUC03 from Imaging source (www.theimagingsource.com) and its acquisition software on the computer, and set AVI format, choose a minimum frame rate (30 frames per second provides sufficient information to analyze trajectories but are insufficient to study the wobble of sperm heads). In the present purpose a frequency of 30 frames per second is adequate



- 2) Set the revolver to X20 magnification
- 3) Pre-focus an already sperm-filled counting cell, the depth of which is either 10 or 20 μm (Leja cell, Makler cell or even a simple Thoma cell). The 10 μm depth is well adapted since it limits the vertical dimension of sperm trajectory, hence preventing focus modification needs during the record. Makler cell is very expensive and reusable, while Thoma cell is much less expensive and also reusable. However one of the problems met when recording, is the difficulty to get clean background at each trial in order to avoid particles that may interfere with the analyses. The single use Leja cells are very useful, although rather expensive, since the chambers are well calibrated and clean allowing rapid experiments in reproducible conditions (cost of one trial = 1 euro). The use of dark field or phase contrast may help improving the quality of movies for CASA analysis

B) Sperm preparation

1) Sample collection

The semen is obtained by gentle abdominal pressure of the male, after wiping the genital area in order to avoid any pollution by the external medium (seawater) and it is collected at the gonopore with a vacuum device (syringe or vacuum tube); semen fractions visibly polluted by urine must be discarded.

2) Dilution protocol

A two-step dilution procedure is recommended in order to avoid sperm aggregation and differential motility trigger:

- The first dilution aims at adjusting the concentration of spermatozoa without triggering motility and / or improving the storage of semen. For this, a non-activating medium (NAM) is used. Generally an iso- or hypo-tonic saline can be used, however, improved media can be designed to assess motility while improving sperm storage for example (see annex).
- The second dilution aims at triggering the motility of spermatozoa. The use of seawater supplemented by 20 mg ml^{-1} Bovine Serum Albumin (BSA) or by 0.2% Pluronic (P1300, Sigma-Aldrich) as an activating medium allows a natural motility, while it prevents spermatozoa from sticking on the counting cell's glass.

As far as we know, a final density after activation of 1 to 2×10^4 szoa μl^{-1} provides an adequate number of motile sperm for motility analysis

3) Dilution of sperm to 1/25 in NAM.

Since sperm may be viscous, a volume of 10 μl sampled with a positive displacement capillary micropipette (*e.g.*, Socorex, Switzerland, or Gilson) would be the necessary quantity for a sufficient precision of the sample. Sperm will be mixed with 240 μl of NAM in a 1.5 ml tube

4) Preparation to activation.

Fill several 1.5 ml tubes with 990 μl activating medium (seawater+BSA). For each trial one tube will be used.

Deposit a 10 μl drop of diluted sperm on the wall of the tube without any contact with activating medium. (At mixing, the drop will be diluted in seawater to 1/100 and the total dilution of sperm will be 1/2500)

The concentration of sperm reported for grey mullet is very variable from 6×10^9 to 2.5×10^{10} szoa ml^{-1} (Sadeghi *et al.*, 2009). Since the latter value corresponds better to what is generally observed in marine fishes, we propose a model protocol based on a concentration of 2.5×10^{10} szoa ml^{-1} . In case of lower concentration of sperm, the dilution protocol should be modified accordingly.



C) Sperm motility record

The principle to get a good record of sperm motility is to control accurately the time of spermatozoa activation. For this, the contact between sperm and activating medium must be immediate and throughout the sample. Sperm should be activated only after microscope pre-focussing. For this, insert an empty and clean observation cell as described previously. To trigger activation, we propose to do the following concomitantly:

- Take the tube containing activating medium with the drop of diluted sperm deposited on the wall and shake it strong only once, closing the tube with one finger,
- Start the video record with the other hand, in order to get a clear and reproducible start point (time 0 = activation)

Then, as soon as possible

- Collect 2 to 5 μl of activated sperm with a classic micropipette,
- Introduce it between slide and cover slip ,
- Fine focus.

This can be done very fast and allows a first observation of motility at less than 10 s after activation. A drift of sperm can occur when the deposited volume is too large. In case this happens, use a smaller volume.

D) Install the plugin CASA Image J

After having downloaded the CASA plugin from Image J site (rsb.info.nih.gov/ij/plugins/), go to plugins->install, then choose CASA in the plugins window and save. The CASA plugin will appear in the list of available plugins (see arrow in **Fig. 1**)

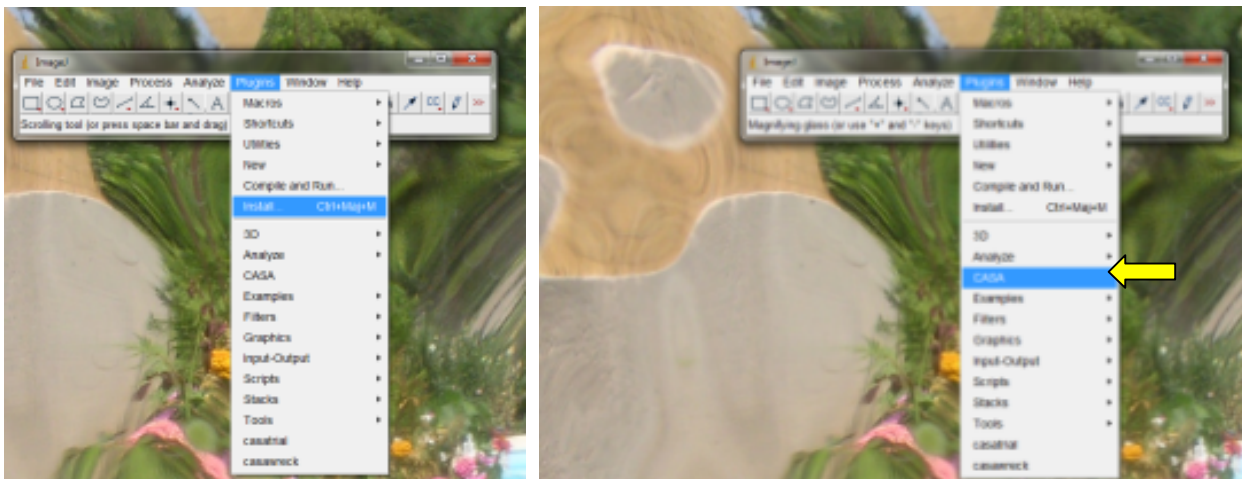


Figure 1 The CASA plug in from Image J.

CASA analysis using Image J

The movie can be directly loaded to image J by simply dragging the icon of the movie to the bottom part of the Image J toolbar, then drop it. However, in case of too heavy movie or problems of unsupported AVI file, it is possible to transform the movie into image sequence using a free software such as VirtualDub, the use of which is the following:



Movie process by VirtualDub: If the movie (AVI file) is too heavy, open VirtualDub, then open the file (green arrow **Fig. 2**) limit the sequence of interest by setting the little landmarks at the right position (in our case since the record was launched at activation, *i.e.*, before the deposition of sample under the objective, the sequence of interest will start at 10 s (300 frames if the video camera was set to 30 frames s^{-1}) and will finish at 3200 frames (red circle)).

Export the data as an image sequence in a previously established new folder (**Fig. 2**, yellow arrow). In this demonstration, the folder was called freshmale5 (**Fig. 2**, red arrow)

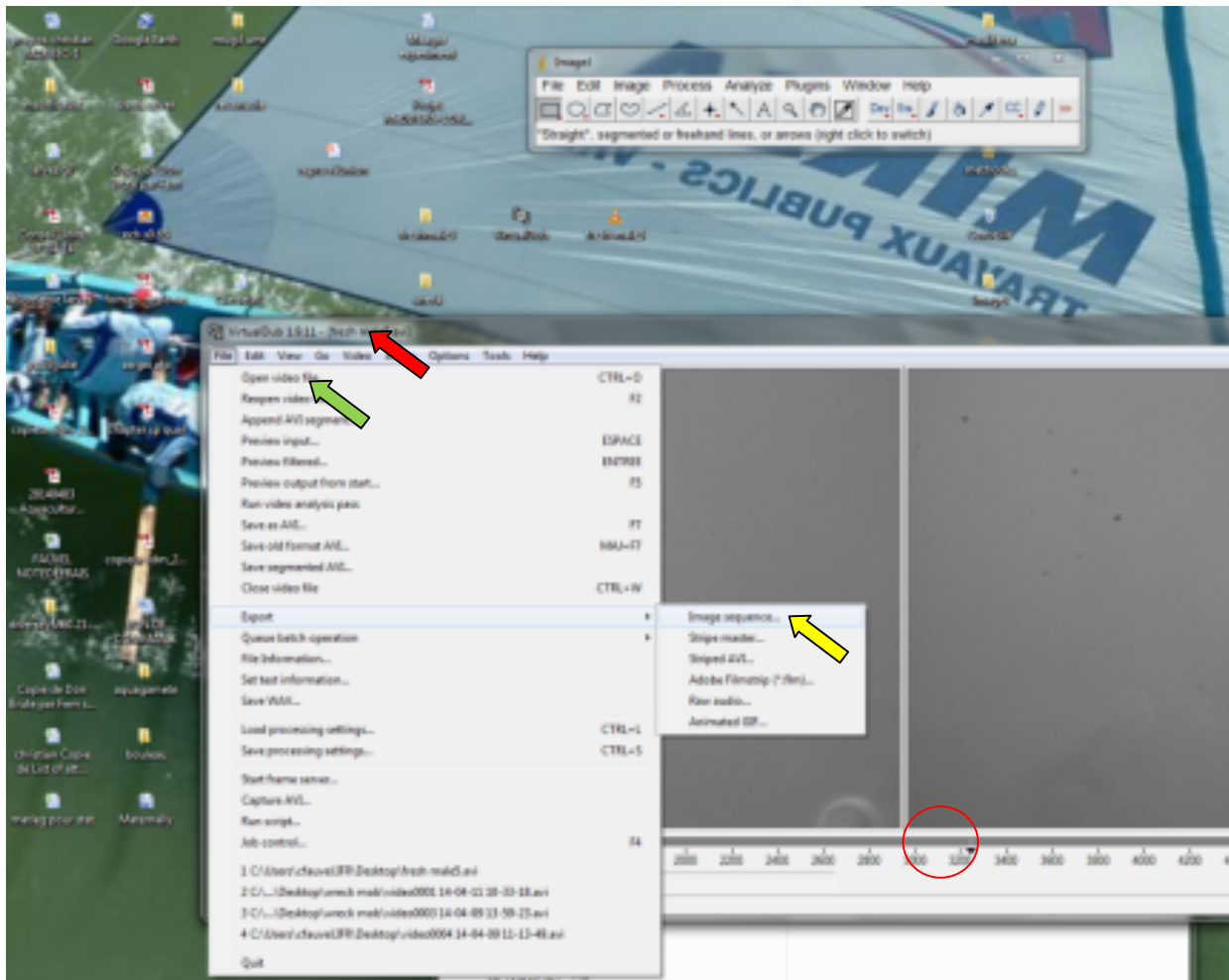


Figure 2 The menu of VirtualDub, which can be used to transform the movie into image sequence. See text for position of arrows.

Movie/sequence process in Image J

1) Sequence length definition

- Open the folder containing the sequence of pictures and just select the first picture by one click, then Import the image sequence according to the path shown in **Fig. 3**.
- Choose the number of images you want to analyze and the starting image of the sequence of interest (for a 2 min motility, sequences of 1 or 2 s, every 10 s would be adapted to appreciate the decrease of motility with time)



In Fig. 4, we select a sequence starting at 10 s. of a movie recorded at 76 frames per seconds and request the treatment of 2 s.

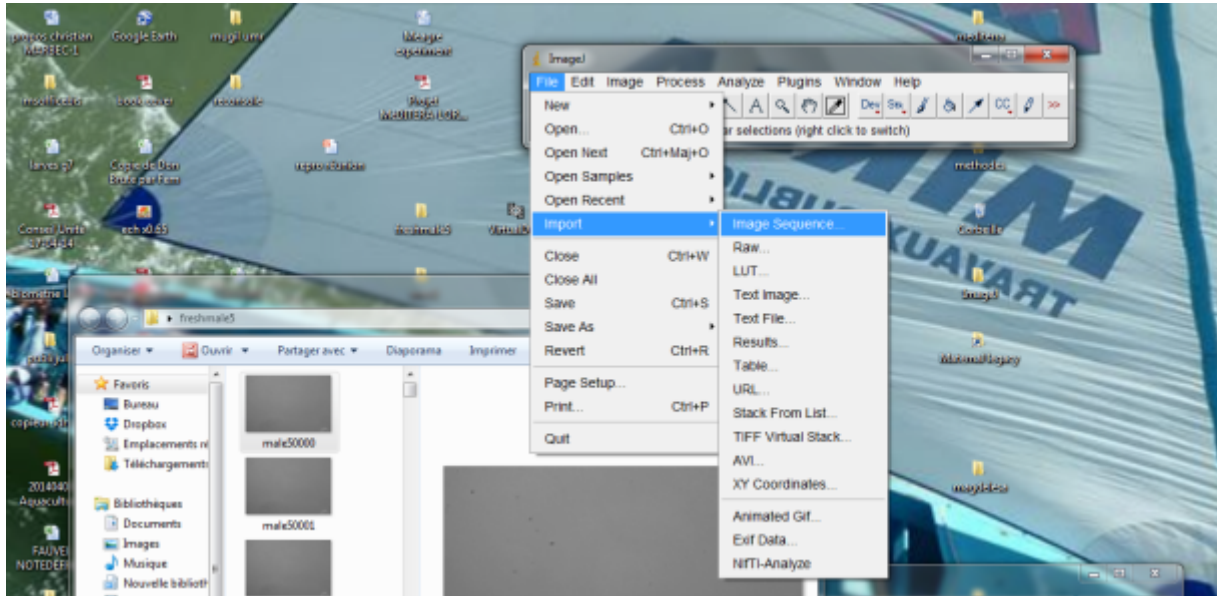


Figure 3 Path sequence for importing the image sequences from VirtualDub.

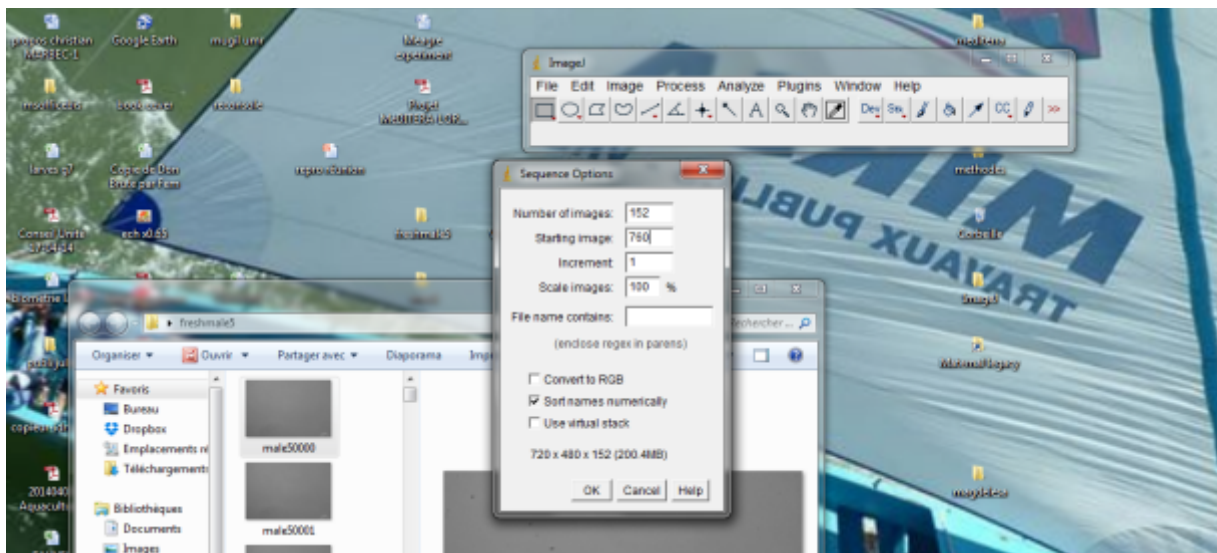


Figure 4 Selecting a sequence starting at 10 s of a movie recorded at 76 frames per seconds and requesting the treatment of 2 s.

Image treatment to define particles of interest (spermatozoa)

- a) Record the process. Since the whole movie may present a similar background it is interesting to set common parameters for the image treatment and CASA analysis of the different little sequences of 1-2 sec describing the motility variations with time. In order to do this, the best option is to build a



macro by a simple record of the successive actions of the treatment using the following path. The different actions will be edited in the window Recorder (arrow) (Fig. 5).

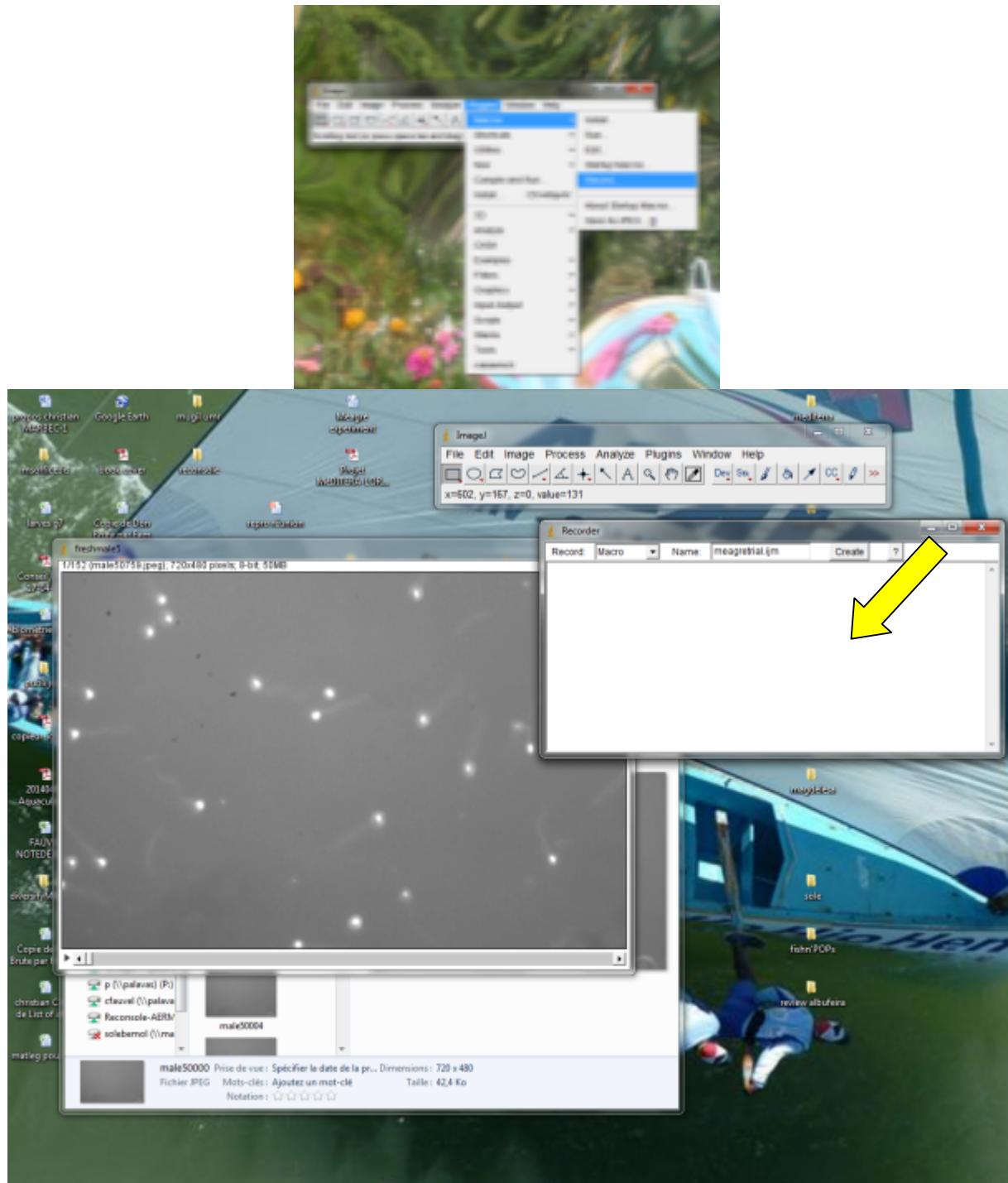


Figure 5 Building a macro by a simple record of the successive actions of the treatment.

b) Image treatment. The aim is to recognize the particles of interest (spermatozoa) and separate them from the background by binarization. The different steps of image treatment are contained under the title “image”.



Step 1: transform the type of image to 8bits (grey levels) according to the following path (**Fig. 6**)



Figure 6 Transforming the type of image to 8bits (grey levels).

Step2: adjust brightness and contrast in order to facilitate the choice of particles (**Fig. 7**)

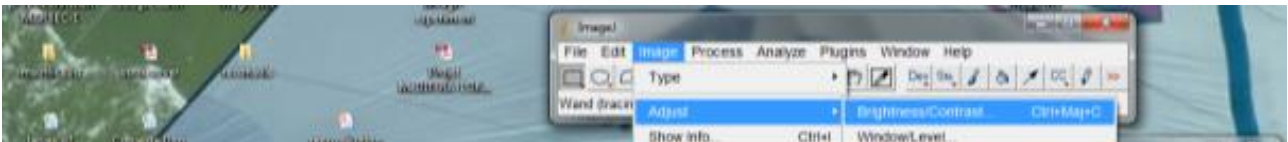


Figure 7 Adjust brightness and contrast in order to facilitate the choice of particles.

Playing with the cursors (yellow arrows), allows to increasing contrast and adjusting brightness so as to obtain a black and white image such as in **Fig. 8**. Note that at this stage the image is still a picture made of different levels of grey represented by the histogram in the B&C window.

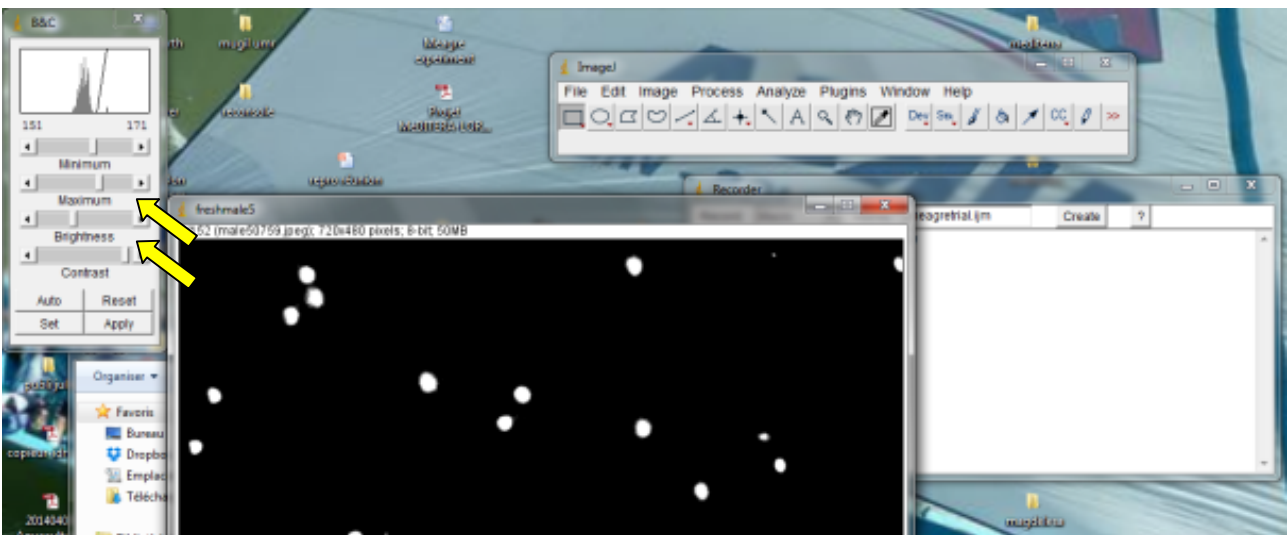


Figure 8 Increasing contrast and adjusting brightness so as to obtain a black and white image using the cursors.



Record the settings for B&C by the successive clicks on set and apply (arrows in Fig. 9).

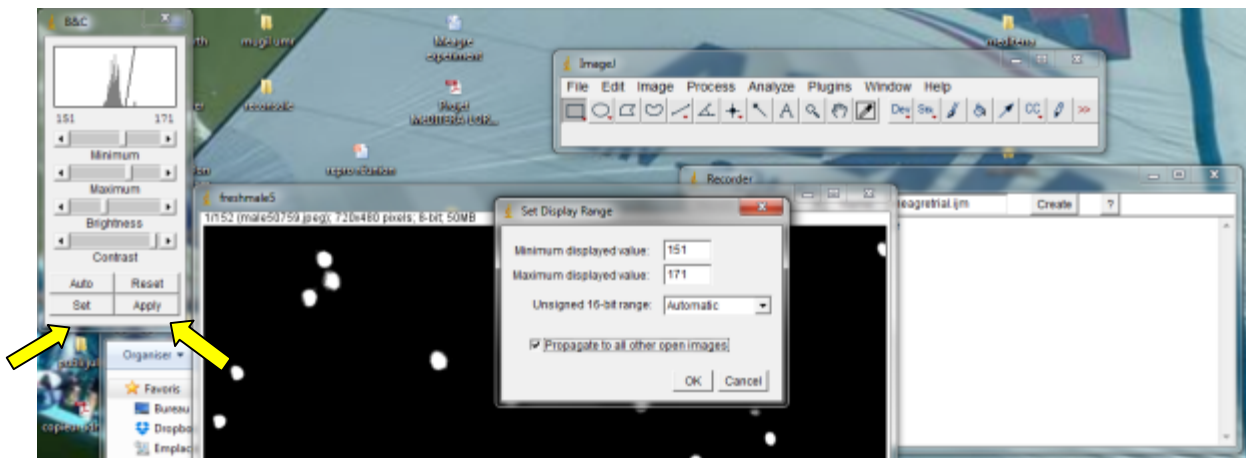


Figure 9 Recording the settings for B&C.

Step3: Separate the particles of interest from the background by the application of a threshold (binarization) with the following path (Fig. 10).

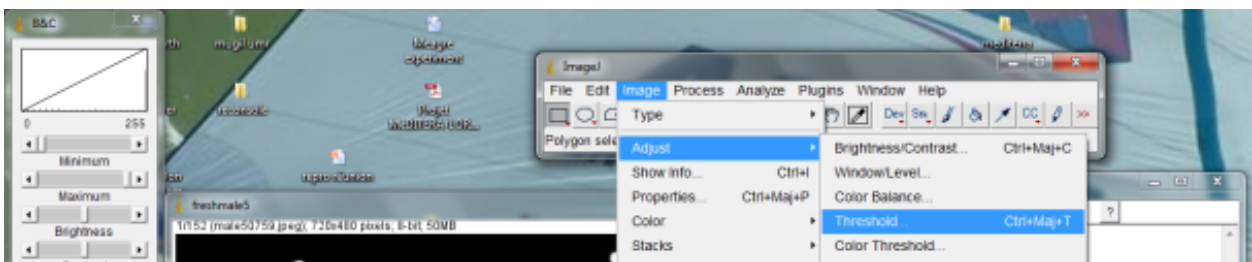


Figure 10 Application of a threshold (binarization).

The system proposes an automatic choice (what is selected as area of interest is coloured in red) but you can modify using the cursors or threshold window (Fig. 11).

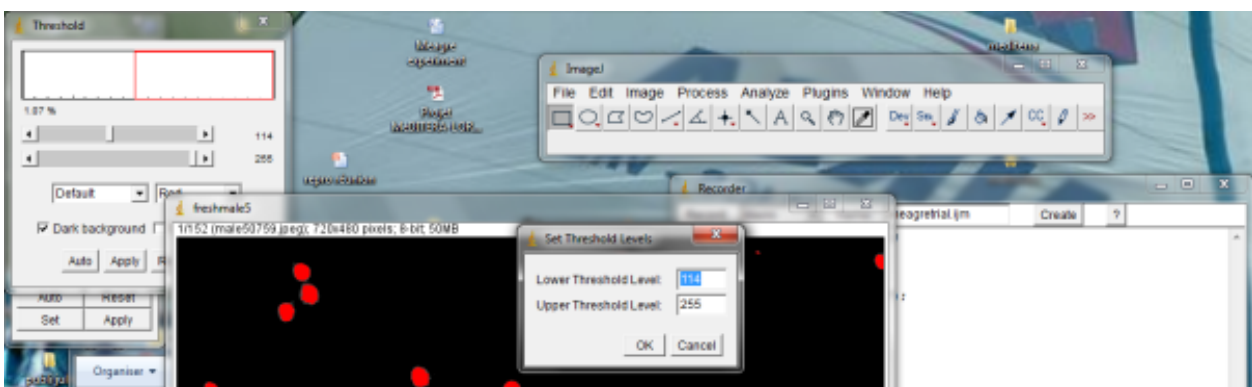


Figure 11 Modifying the auto choice using the cursors or threshold window



Set and apply the settings of threshold. Don't forget to click on "calculate for each image" (Fig. 12).

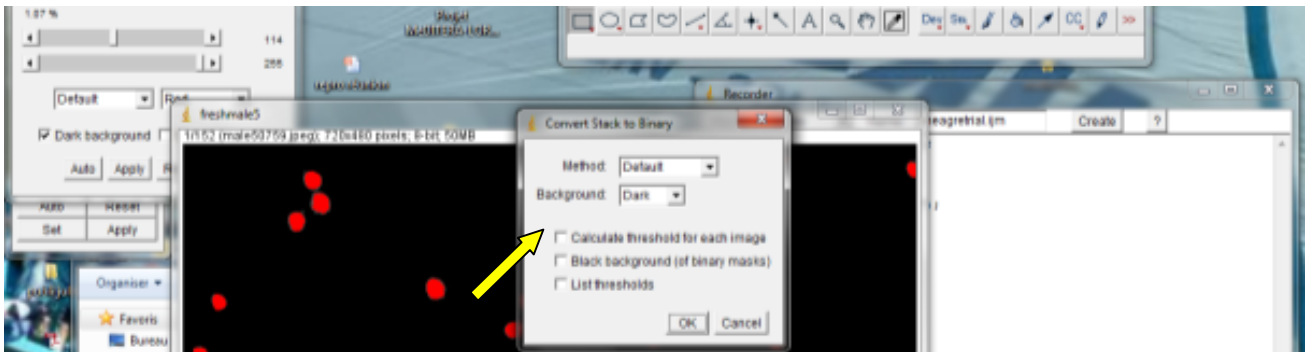


Figure 12 Calculating each image.

As a result, you obtain black particles (spots to be studied in black and background in white). Moving the cursor (arrow) allows looking at all the pictures of the sequence (Fig. 13)

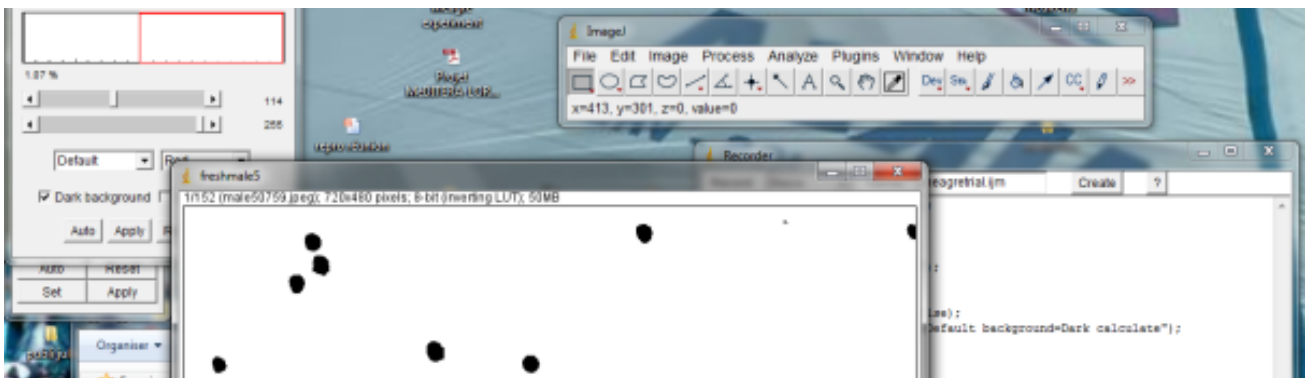


Figure 13 Moving the cursor (arrow) allows looking at all the pictures of the sequence.

This is the last step of image treatment before the analysis of particle movement (CASA).



Analysis of motility (CASA)

Click on plugins icon, then open the CASA dialog box (**Fig. 14**).

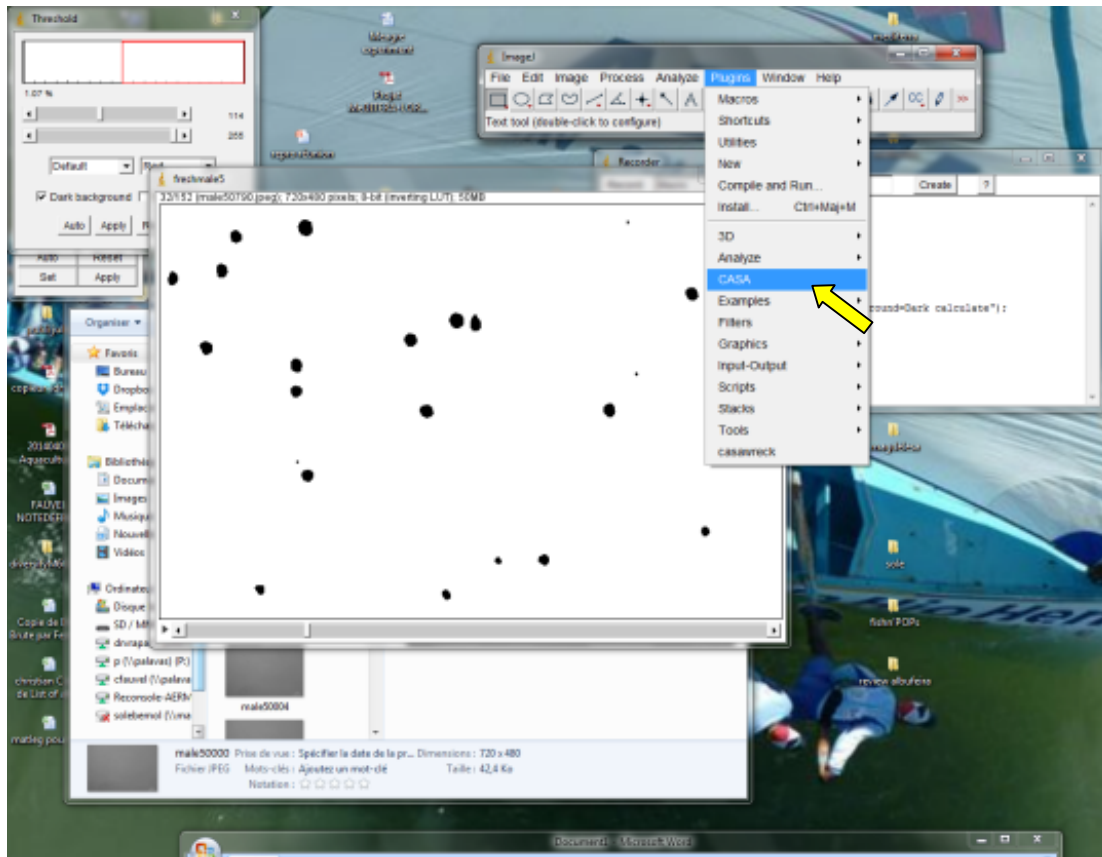


Figure 14 Opening the CASA dialog box.

The system proposes different parameters to be adjusted for a realistic assessment of motility. The different parameters are detailed in the original document of Wilson Leedy and Ingermann (2007), but let us go through the most important in **Fig. 15**.

- Setting a low but no null number of pixels for minimum may allow eliminating small moving particles which must not be assimilated to spermatozoa
- Can be set at a high number of pixels if the background is clear for all the images of the sequence
- Is quite important since it allows you to set the minimum duration for a representative track in the present case, 8 frames represent around 0.1 second a too long minimum duration may limit the number of spermatozoa taken into account if they quit the field during the sequence.
- Needs some trials because it depends on the velocity of sperm. The value indicates the area in which the system will search for the position of a given particle in the next frame. A too high value can drive the system to jump from one spermatozoa to another provoking erratic trajectories.
- (f) and (g) allow to precise what is considered as a motile spermatozoa. It can be useful to eliminate the artificial motility generated by drift when it occurs.
- Is very important and represent the frame rate you set to the video camera Will give the calibration of the system in relation to the magnification you used
- (s) (t) (u) allow to choose the data you want to get from the analysis.

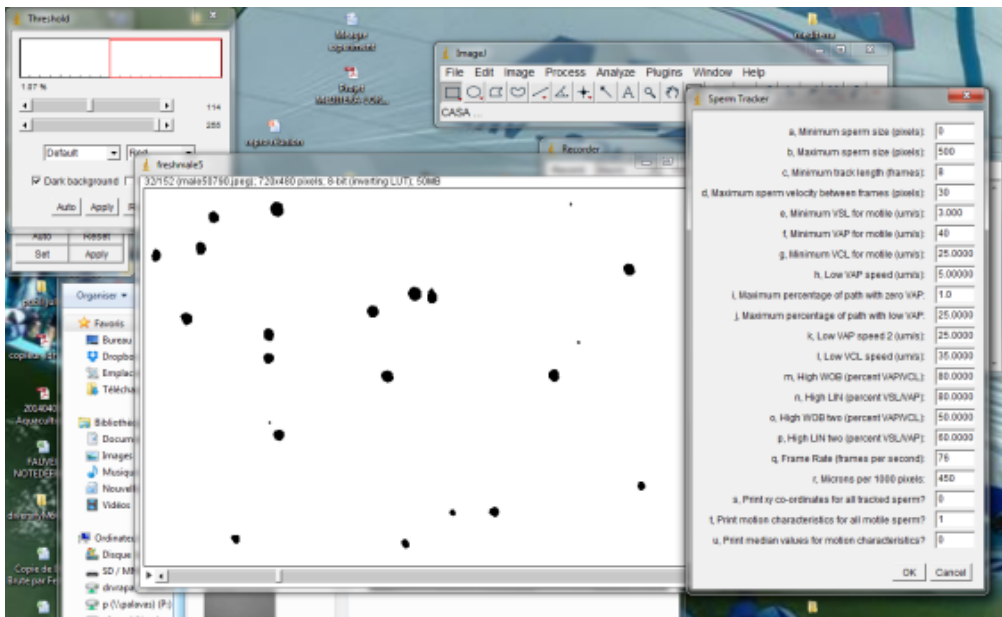


Figure 15 The different parameters are detailed in the original document of Wilson Leedy and Ingermann.

When the parameters are set, a simple click on Ok launches the whole CASA analysis, which generates a result table and a window where representations of the tracks are drawn. (Note that when magnifying the image, some tracks appear in grey colour indicating that the particle was considered as immotile according to the settings. (Fig. 16).

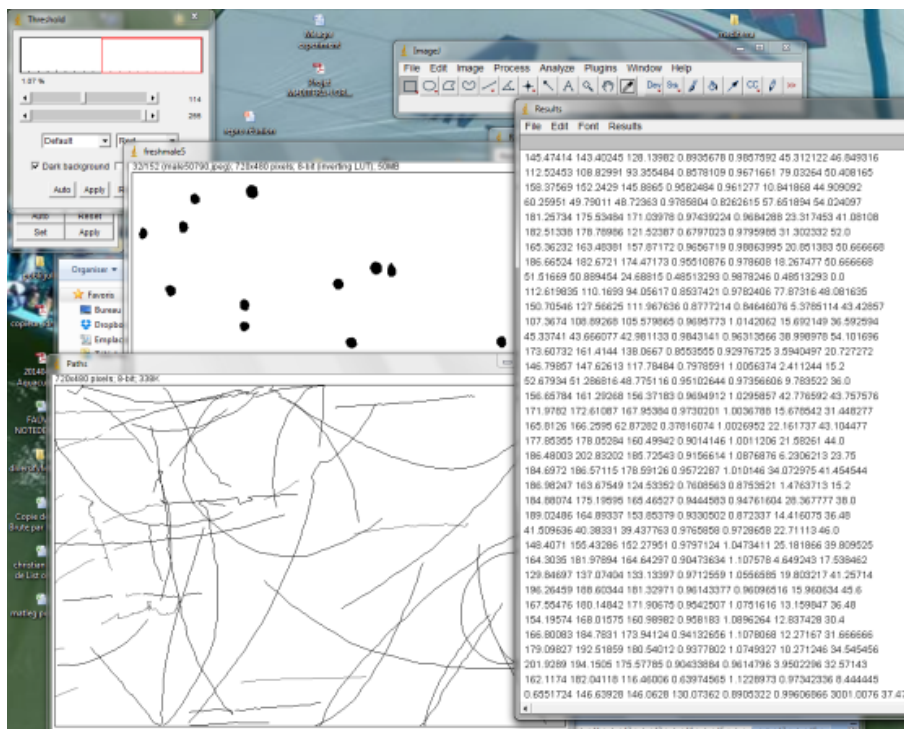


Figure 16 CASA analysis, generating a result table and a window where representations of the tracks are drawn.



Then select and save the data, which will be recorded as excel file (**Fig. 17**). Save the macro after having selected all (**Fig. 18**).

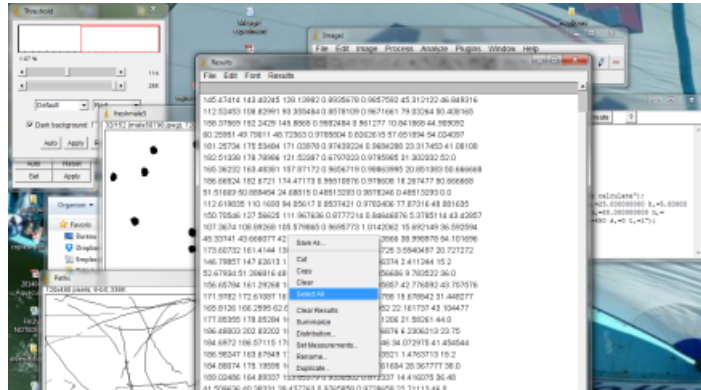


Figure 17 Selecting and saving the data, which will be recorded as excel file.

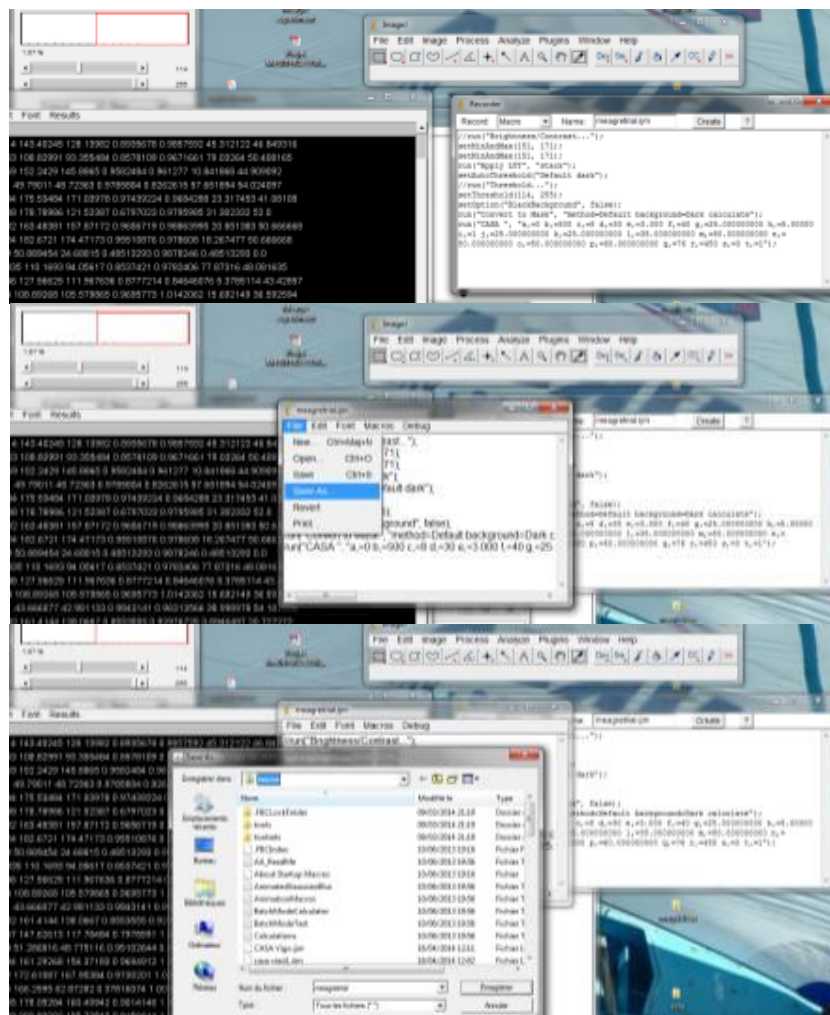


Figure 18 Saving the macro.



The first phase of motility one or two seconds is now studied then choose the following sequence (for example at 10 seconds (drag and drop the movie, then choose starting image and number). Install the macro you just recorded according to the following procedure **Fig. 19**. Finally launch the new analysis by clicking on the name of the macro (**Fig. 20**). Repeat the operation at any time point you desire in relation to the total duration of motility.

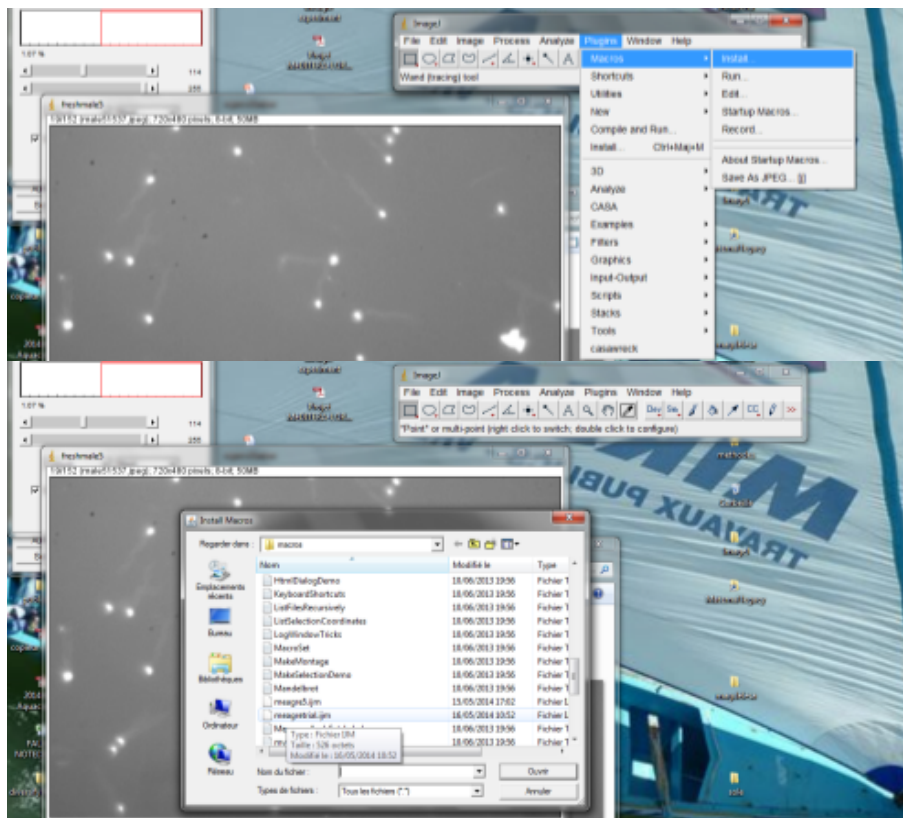


Figure 19 Installing the macro.

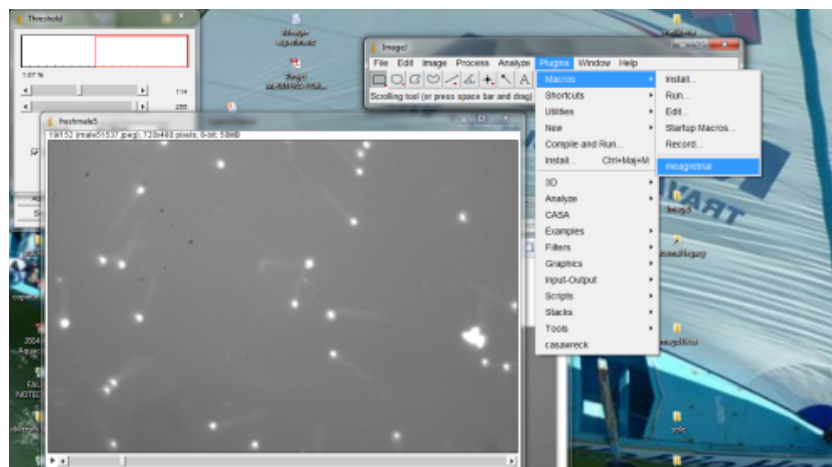


Figure 20 Launching the new analysis by clicking on the name of the macro.



Application of CASA to grey mullet sperm.

Sperm preparation and motility record

During November 2014), 6 sperm samples were collected from sexually mature grey mullet males held in the P4. IOLR facilities in Eilat. According to the procedure described above, different dilutions of sperm were tried. The gametes were activated by the addition of seawater and the movement of spermatozoa was recorded (10 sec after activation; frame rate of 100 FPS). The movies were either studied *in situ* or sent by Dropbox to Ifremer (Palavas, France) in order to inter-calibrate the analysis through mail exchanges.

A dilution of 1/1000 was chosen as for the analysis this dilution provided approximately 80 spermatozoa in the field of the microscope (X 20 magnification). This configuration was a good compromise between the total number of observations and the quality of observation (no errors of jumps from one sperm to another sperm in the traced trajectory were observed with the 1/1000 dilution).

The quality of these first trial movies allowed the CASA protocol to be established, but as emphasized in **Fig. 21**, these were not yet optimized due to the settings of the microscope. The upper and lower right corners were blind zones, making impossible the analyses in these areas.



Figure 21 Heterogeneity of background. From an original image (left) increasing contrast allow to showing blind zones(center). Maximum contrast and adapted brightness separate spermatozoa from background (right).

CASA Analysis

The movies were processed and analyzed once specific macro was established and recorded on the first second of the sequence. As an example, the macro corresponding to the movie shown in **Fig. 21** was the following:

```
//run("Brightness/Contrast...");
run("Next Slice [>]");
run("Next Slice [>]");
run("Next Slice [>]");
run("Next Slice [>]");
run("Previous Slice [<]");
run("Previous Slice [<]");
setMinAndMax(158, 160);
run("Apply LUT", "stack");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(128, 255);
setOption("BlackBackground", false);
```




```
run("Convert to Mask", "method=Default background=Default calculate");  
run("Fill Holes", "stack");  
run("CASA ", "a,=0 b,=500 c,=50 d,=8.00000000 e,=3.000 f,=20.00000000 g,=25.00000000 h,=5.00000  
i,=1 j,=25.00000000 k,=25.00000000 l,=35.00000000 m,=80.00000000 n,=80.00000000  
o,=50.00000000 p,=60.00000000 q,=100 r,=1075.00000000 s,=0 t,=1");
```

The macro was applied to a 1-s sequence every 10 s until all sperm movement ceased. The trajectories of the spermatozoa are shown in **Fig. 22**.

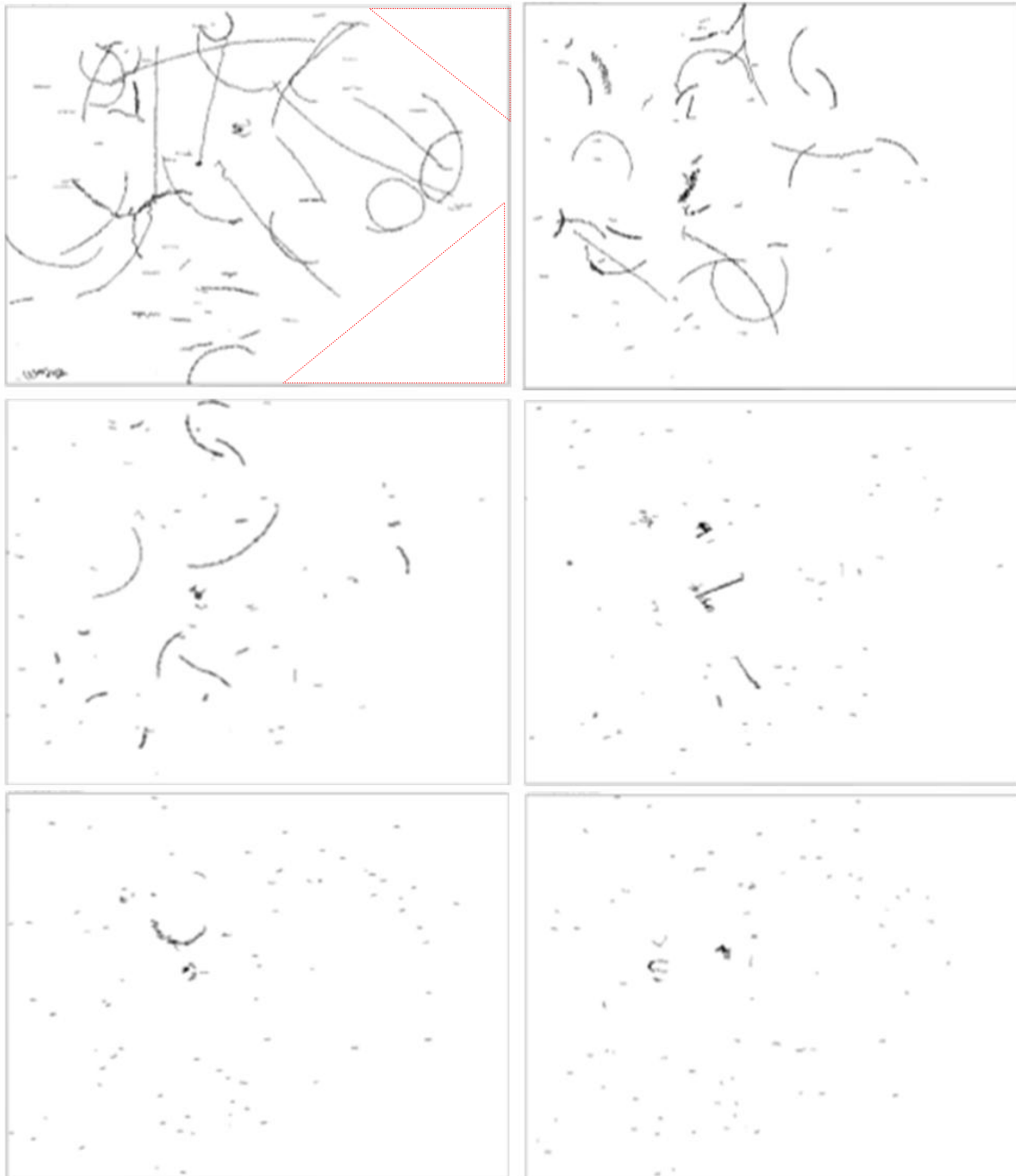


Figure 22 Trajectories of around 80 spermatozoa at different times after activation. Red forms indicate the blind zones due to heterogeneous background of the movie.



In the current case, the motility of grey mullet decreases with time as is the case for all fish species and the duration was limited to 50 seconds. The initial percentage of motile spermatozoa was around 50% and quickly decreases to zero after 70 seconds (Fig. 23).

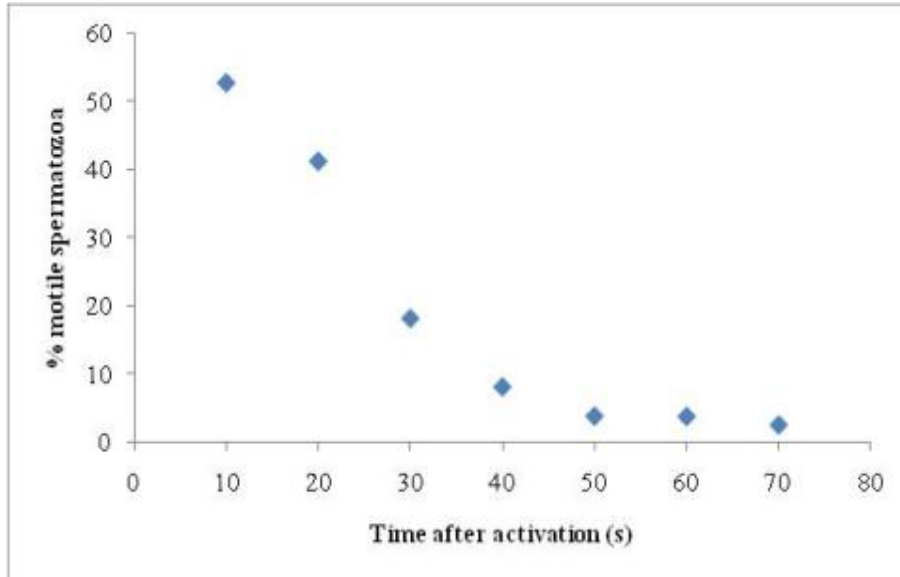


Figure 23 Variation of grey mullet sperm motility assessed by the percentage of motile sperm with time after activation.

Whereas the number of motile sperm decreases, the mean speed of spermatozoa also drops with time (Fig. 24). The variability of velocity remained high along the studied sequence but the higher velocities decreased strongly. The comparison of means by ANOVA revealed that the only significant difference in velocity was observed between the velocity at 10s and all other time points.

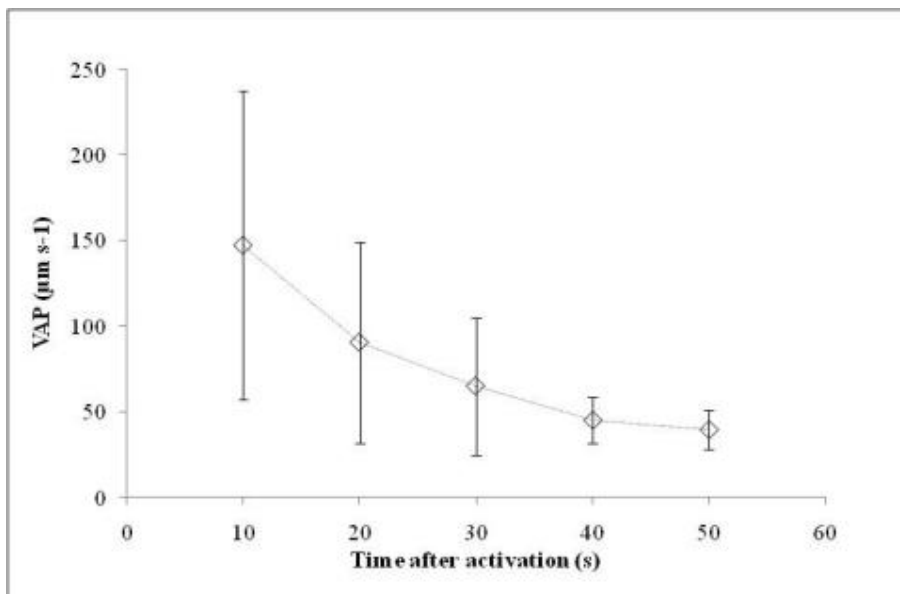


Figure 24 Modification of Average Path Velocity (VAP) of grey mullet sperm with time error bars illustrate the standard deviation.



Finally, the calculated linearity objectively assessed the variations of trajectories during the spermatozoa propulsive movement. During the first 30 seconds after activation the majority of spermatozoa swam in a straight line, then the track bent progressively as illustrated by the decrease of linearity (**Fig. 25**).

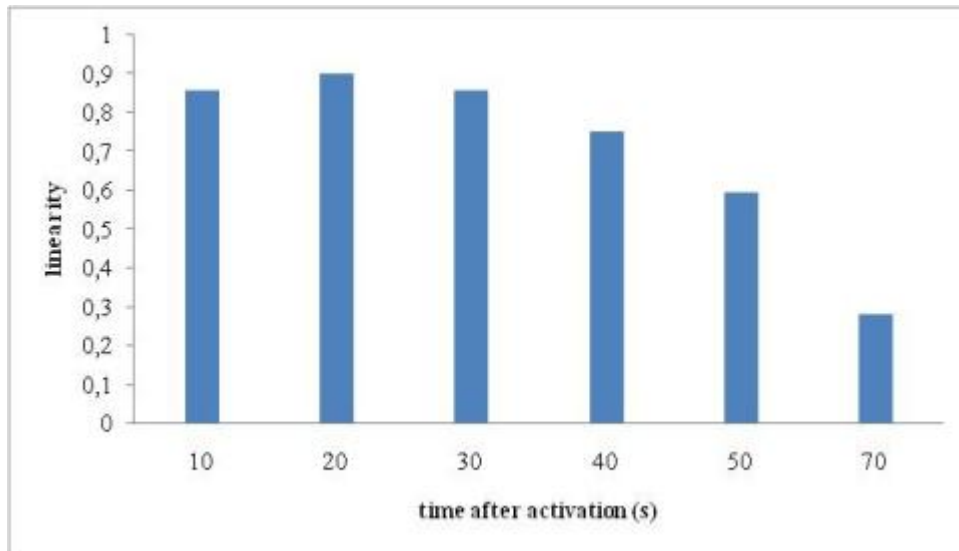


Figure. 25 Modifications of the trajectories of grey mullet sperm expressed by the linearity.

***In situ* implementation at the P4. IOLR facilities and the system specs and properties**

The setup of the P4. IOLR CASA system, as well as optimization of dilution, activation and storage protocols were conducted by Dr. Tzilhav Shem-Ad. The system included a >120 frames per s (640x480 resolution) cmos camera (UI-3250LE from IDS Imaging Development Systems GmbH) connected via USB3.0 to an I7 microprocessor with 64-bit architecture equipped with 1TB SSD hard drive. These specs ensures that information resulting from capturing and recording of activated grey mullet spermatozoa will be transferred onto the computer's hard drive with virtually zero latency. Capturing and recording of activated grey mullet spermatozoa was done by using a software provided by the camera's manufacture. The camera was connected to a ZEISS dark field microscope via c-mount adaptor.

Adjustment and calibration of the activation, dilution and storage protocols.

Sperm were collected from grey mullet as described earlier. In order to dilute samples into a desirable dilution factor (1:1000), where a total of 80-145 sperm cells is achieved in the video field for analysis; first, 2 μ l of the collected sperm was added into 198 μ l of Non Activating Medium (NAM) resulting in a 1:100 dilution of the sperm samples. NAM is a modified L15 medium (Sigma-Aldrich, IL), with the exception of adding 20 mg/ml BSA and pen-strep antibiotic. Second dilution was done at the time of activation by adding 10 μ l from 1:100 diluted spermatozoa samples into 1.5 ml tubes containing 90 μ l of filtered (0.2 microns filter) seawater. The seawater salinity was ~42‰ and pH was 7.3-8.0. Spermatozoa were measured for motility 10 s after activation. The original sperm samples, as well as 1:100 diluted samples were preserved at 4°C.

Adjustments and calibrations of the ImageJ analysis protocols.

First, we adjusted the files background, brightness and contrast to a level in which ImageJ software recognized almost all the spermatozoa in the captured screen (**Fig. 26-29**). This was a crucial adjustment since it resulted in analyzing almost all of the cells in the samples (**Fig. 30**). Second, we recorded our steps



onto a macro java file so that each recording we made could be analyzed by the same manner and with consistency (supplementary 1). Finally, by using the CASA plug in parameters, as described by Dr. Christian Fauvel, we obtained the activated spermatozoa motility parameters and a comparison to Dr. Fauvel's analysis was made in order to inter-calibrate the system.

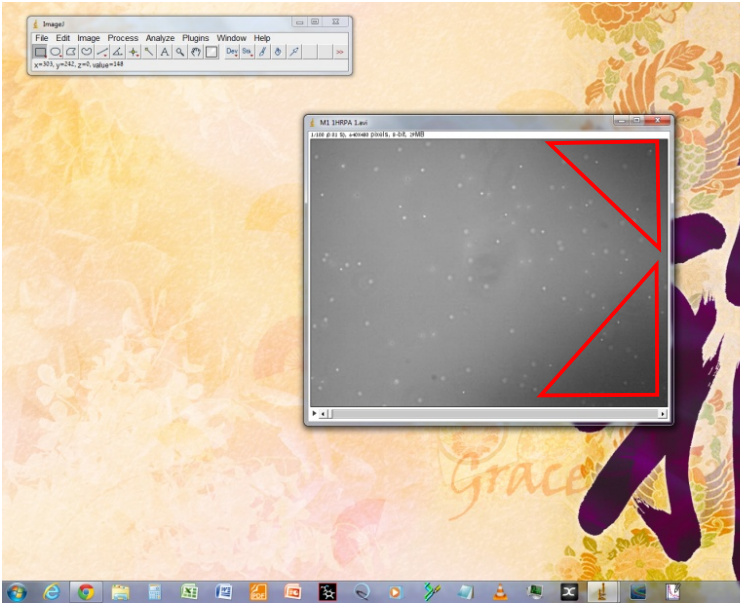


Figure 26 An original imported activation AVI file print screen output. The image demonstrates a unhomogenized background issue (denoted by red triangles).

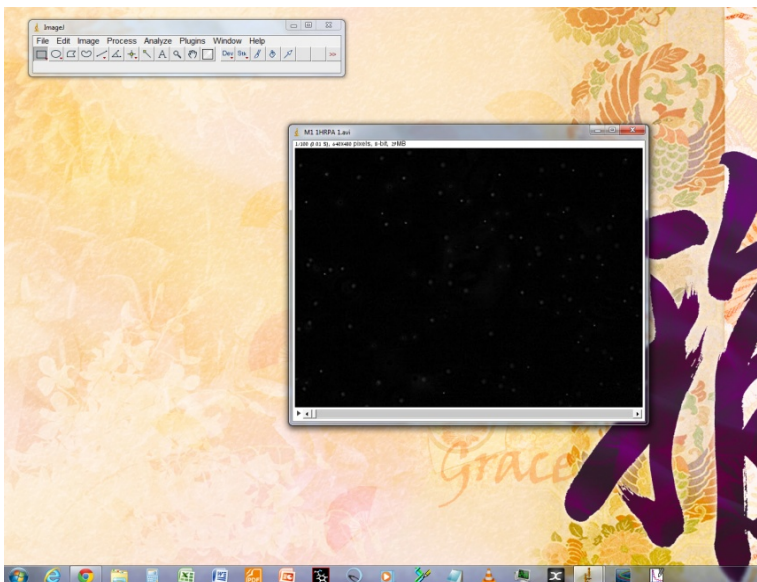


Figure 27 Eliminating the uneven intensity values of the image's background pixels. The image displays a fairly homogenized pixels intensity values after applying the following commands: process-subtract background-subtract background by a factor of 50 pixels.

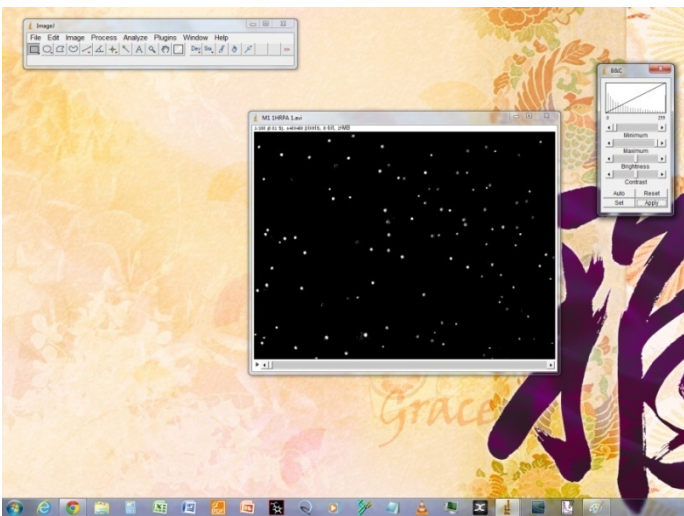


Figure 28 Enhancing the separation between particles (spermatozoa) and the image's background. An additional brightness and contrast manipulations were required for achieving an overall separation.

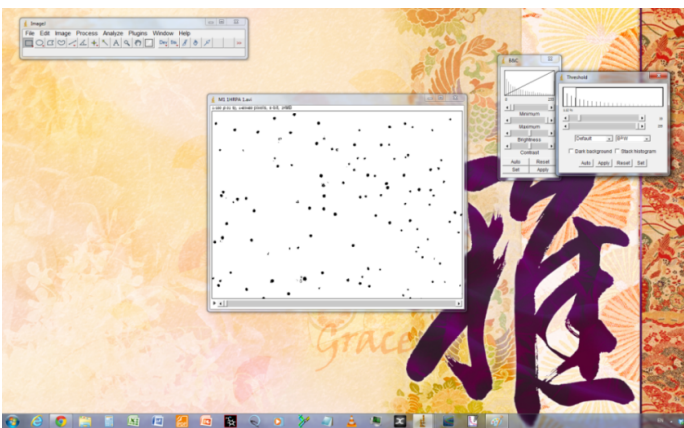


Figure 29 Adjusting the image's threshold values enables the CASA plugin to detect and analyse particles. Here we can see that all the image's areas were taken into considerations. Areas that once were dark and hidden will now be analysed by the CASA plugin (denoted in red triangles).

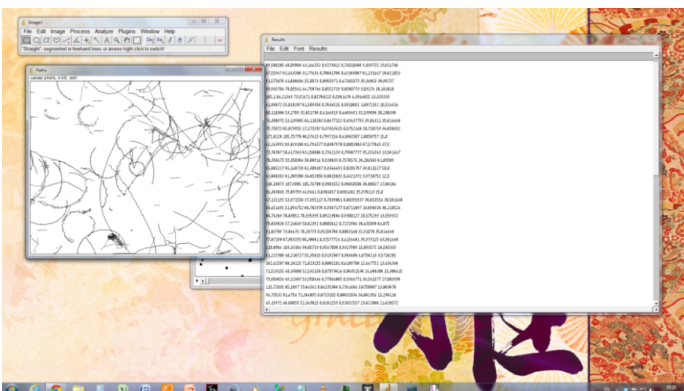


Figure 30 View of the tracks resulting from IOLR protocol (**Annex 1**) showing the ability to take spermatozoa moving in the dark parts of the background into account.



Annex 1: "Analysis AVI activation files.ijm macro" file

```
run("Subtract Background...", "rolling=50 stack");
//run("Brightness/Contrast...");
run("Apply LUT", "stack");
setAutoThreshold("Default");
//run("Threshold...");
setThreshold(29, 255);
setOption("BlackBackground", false);
run("Convert to Mask", "method=Default background=Light");
run("CASA ", "a,=0 b,=500.000000000 c,=8.000000000 d,=30.000000000 e,=3.000
f,=40.000000000 g,=25.000000000 h,=5.00000 i,=1
j,=25.000000000 k,=25.000000000 l,=35.000000000
m,=80.000000000 n,=80.000000000 o,=50.000000000
p,=60.000000000 q,=100.000000000 r,=340.000000000 s,=0 t,=1");
```

Conclusion and perspectives

As programmed in the DOW, a method for an objective assessment of sperm quality through the analysis of motility was implemented for grey mullet (*Mugil cephalus*). The preliminary analyses show that grey mullet sperm presents a pattern of motility similar to that of seabass (*Dicentrarchus labrax*) or rainbow trout (*Oncorhynchus mykiss*) in terms of velocity as well as duration which lasts less than one minute. The objective of this deliverable was to provide a tool for the objective comparison of sperm quality of male grey mullet subjected to different hormone-based treatment. The CASA tool, has been transferred through a short term scientific mission from Israel to France where it had been possible to train a scientist of IOLR, applying the general protocol to currently mature meagre males during late spring. Then during autumn, grey mullet sperm were activated under video camera. To further attest trainee's qualification, analyses of the resulting movies were carried out, in parallel by the Ifremer and IOLR teams, and compared.

Our preliminary results, indicate that the major bio-technical settings dilution of sperm, quality of activation and mastering of video recording have been determined and can now be used in experimental protocols. It was recommended that there is a need for a better quality of the background of the pictures. To achieve this, care will be needed in lens cleaning and the settings of the video microscope which is a major issue in this type of experiment.

Since fish sperm quality is usually assessed through subjective observations such as motility classes, and motility duration estimated *de visu* preventing precise statistical analysis, the current results will help contrasting results and improve assessment of the effect of different treatments on reproductive performances of grey mullet males.

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Deviations: No deviations



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