Journal of Applied Ichthyology

J. Appl. Ichthyol. 28 (2012), 1013–1016

ISSN 0175–8659

Received: May 26, 2012
Accepted: September 6, 2012
doi: 10.1111/jai.12070

Technical contribution

Modifications to the IMAGEJ computer assisted sperm analysis plugin greatly improve efficiency and fundamentally alter the scope of attainable data

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Summary

Computer assisted sperm analysis (CASA) has greatly enhanced the quality and quantity of data that can be collected on spermatozoa movements. Open source IMAGEJ software allows any researcher to have access to such analyses, but CASA systems in general are not designed to ably process large numbers of video sequences. Here we introduce a modified IMAGEJ CASA_automated plugin that fundamentally enhances efficiency and will allow higher quality research to be conducted with no extra post-microscope effort.

Introduction

Animal sperm were first discovered in the 1670s (Leeuwenhoek, 1678), but quantitative measures of sperm movement began only decades ago when researchers started recording video of microscopic observations. These data were initially obtained by attaching a clear acetate sheet to a video screen, advancing the video sequence one frame at a time, and manually determining sperm movement patterns. The approach was radically altered with the development of computer assisted sperm analysis software (CASA) in the 1980s. This was a major step forward as sperm movements could be determined more objectively, and once video was acquired the time required to extract data was reduced. A major constraint of this advancement however was availability. The extremely high price of such software meant few researchers had access to it.

Another significant milestone in the assessment of sperm movement patterns came in 2007 when Wilson-Leedy and Ingermann (2007) developed a CASA software package that worked as a plugin to the United States National Institutes of Health (NIH) IMAGEJ software. This being open source and free allows any research laboratory to have access to CASA software, and creates the possibility of standardization. Although the IMAGEJ plugin is available to anyone, as with other CASA software (e.g. ISAS, Hamilton Thorne) it is not very efficient to use for a large number of video sequences, as post-video-acquisition data processing time is considerable. This common problem creates limits on the complexity of experimental designs, number of animals used, or numbers of replicated microscopic observations made. For example, in human clinical evaluation sperm are generally checked under one set of conditions. However with external fertilizers one might wish to evaluate sperm movement under different situations. Adding different levels of more than one environmental variable (e.g. pH, salinity, temperature), with tests of variability among males (e.g. dominance, ages, family groups) and among populations quickly creates a massive data processing problem. Here we introduce a modified CASA_automated plugin for IMAGEJ with greatly improved efficiency, which fundamentally alters the scope of attainable data. We predict that this will be major advancement for research on sperm movement.

Materials and methods

Procedures required to use the IMAGEJ CASA plugin of Wilson-Leedy and Ingermann (2007)

A vital benefit of the IMAGEJ CASA plugin is that it is robust. We use fish sperm as an example to describe techniques used, although the current limitations of CASA and the improvements we show in this paper would apply to any taxa. The exact technique used by researchers to record movements of fish sperm varies. For example, one can take a small semen sample, place it on a microscope slide, add water then a coverslip, put the lens in place, and focus; or using an inverted microscope, one can mix semen and water in a tube and add a drop to a slide without needing to use a coverslip or move lenses. Other combinations of the above are also employed by different laboratories. Importantly, the various techniques used to activate sperm and get them in focus on a microscope slide does not affect video processing in IMAGEJ as long as one uses a microscope setup that produces either a bright white sperm head against a dark background, or a dark sperm head against a light background.

For illustration, let us assume a standard phase contrast microscope is used with a camera that will record video at 200 frames s^{-1} to a computer hard drive. Water is added to a slide, a small amount of semen is mixed with it, a coverslip is added, and the objective lens is moved in place and focused. With practice one may be able to get usable video within 10 s of the sperm being activated once they hit water. With some careful planning or some help one could start recording video as soon as the sperm contact water, and for this example let us assume 41 s of video is recorded of which the last 31 s is useable. Assume for now that the only thing of interest is sperm movement at the earliest time possible (most biologically meaningful) which is at 10 s post-activation. A 0.5 or 1.0 s time period is usually used to quantify sperm motion, and thus for this example movement will be

Table 1
Procedures required to process sperm video using the IMAGEJ CASA plugin of Wilson-Leedy and Ingermann (2007)

Step	Procedure
1	Extract frames: from the 41 s of video (8200 frames) extract the appropriate frames to analyze using other software before importing into IMAGEJ. In our example this would be frame 2001 to 2100 (10.0–10.5 s at 200 frames s^{-1}). Save this as an avi file.
2	Import avi file into IMAGEJ
3	Threshold the images: highlight the sperm heads (these can be white against a dark background or dark against a light background, it only affects the threshold value used), and remove the background. This produces black dots (sperm heads) on a white background for the CASA plugin.
4	Run the plugin on these 100 frames: previously decide what settings to use in the plugin (species and experiment specific).
5	Examine results output: depending on what is asked for, one can either get the mean motion characteristics of all motile sperm in the video, or a row of data for each motile sperm and a row of the means. One would need the means row to get the counts (the plugin counts dots the researcher sets parameters to define as sperm, but only gives data for sperm that are deemed to be moving, that is non-moving sperm do not give velocity = 0). Importantly the plugin does not label the results as being from a particular fish, treatment, replicate, etc.
6	Copy the text results to a text editor
7	Import results into a spreadsheet
8	Label each row of results: the plugin does not label the output; it has to be done in a spreadsheet identifying each row of data as being from a particular treatment, fish, replicate, and time post-activation. If only interested in the means, then one has to label all of these columns for one row of results each time the plugin is executed. If one wants to get data on individual sperm (useful) to average afterwards, there may be hundreds of rows to label for each video sequence analyzed.
9	Separate individual sperm from means data: if outputting the individual sperm movements and the means row, they need to be separated into different worksheets for further analysis (they contain different numbers of columns and do not line up in a meaningful way).
10	Repeat for the next video sequence and continue repeating 500 times: keep all of the plugin parameters the same for each video analyzed in order to get comparable results. Examination of a wide variety of videos from the experiment ahead of time will allow one to choose appropriate parameters. For the example from Purchase and Moreau (2012) one would need to repeat all of the above 500 times to get sperm data just at 10 s post-activation.

Table 2
Procedures required to use the modified IMAGEJ CASA_automated plugin of Purchase and Earle

Step	Procedure
1	Label video from each microscope procedure with an appropriate file name: each file name must contain meaning. For example, M_1_4_2.avi being population = M, fish = 1, treatment = 4, replicate = 2. All of these files must be placed into the same folder. In our example there are 500 files, each 41 s long.
2	Start the "Folder_analysis.ijm" macro: this macro will link to the CASA_automated plugin. Three dialogue boxes will open: 1) set the sperm parameters as with the original IMAGEJ CASA plugin, 2) set which video frames to import for each analysis, 3) tell the plugin what folder all of the files are in. The plugin will do everything else automatically. It will open the first file, extract frames 2001–2100 (10.0–10.5 s), apply threshold, run CASA, label each row of the results with a new column containing the file name followed by _10 (for 10 s). It will then go back to that file, open frames 3001–3100 (15.0–15.5 s), apply threshold, run CASA, and label the results as above coding time as 15 instead of 10, and bin the results with the first group. It will repeat this process for each set of frames that have been setup to be analysed within that 41 s of video. Next, the plugin will automatically go to the next file in the folder and repeat the same process. It will repeat this automatically for all 500 files. Depending on processing speed, the 3500 analyses of Purchase and Moreau (2012) could take the computer several hours.
3	Split the identifiers in the results output: open the single results file in a text editor and split the identifier column into segments by searching for underscore and replacing them with spaces (e.g. M_1_4_2_10 will become M 1 4 2 10).
4	Import results into a spreadsheet
5	Separate individual sperm from means data: if outputting the individual sperm movements and the means row, they need to be separated into different worksheets for further analysis (they contain different numbers of columns and do not line up in a meaningful way). Every sperm is a separate row, has motion characteristics but is also labelled as to fish ID, treatment, time post-activation, etc. The data are now usable but in a relational database like MS Access one can link the mean row of data to the individual sperm rows of data in order to be able to assign the counts and% motile from a specific video sequence to the individual sperm characteristics.

determined from 10.0 to 10.5 s post-activation. The procedure for obtaining data over this time period with the Wilson-Leedy and Ingermann (2007) IMAGEJ CASA plugin is shown in Table 1. However, obtaining motion characteristics for other time periods, treatments, or fish would require repeating the entire process over again and again. The desk time required is substantial and the repetition would likely result in some coding errors. For instance, in an experiment on Atlantic cod sperm, Purchase et al. (2010) used 12 fish, four treatments and three replicates, and quantified sperm motion at four time periods post-activation. Post-experiment manual processing time took >100 h using the ISAS software, and that experiment with 576 comparisons (144 microscope procedures × 4 time periods) was much less intense than the 3500 comparison experiment by Purchase and Moreau that is described below. We have developed a solution to this limitation.

Our modified plugin does not alter the mechanics of that developed and tested by Wilson-Leedy and Ingermann (2007) but rather builds onto it. There are two important limitations that we sought to address: (i) in the original plugin each batch of video frames to be analysed has to be imported manually, threshold values applied, and the plugin executed, and (ii) the results output from the plugin are not labelled to

identify each sperm as belonging to a certain fish, treatment, or replication. We have modified the original plugin to address both of these issues, and illustrate the power of the alterations below.

Results

Procedures required to use the modified IMAGEJ CASA_automated plugin of Purchase and Earle

We start with the same 41 s of video as described above. As indicated useful data exist for the last 31 s but we are interested in motion from 10.0 to 10.5 s. The new plugin will do nearly all of the steps described in Table 1 in one step. It will open the avi file containing the 41 s of data, extract the appropriate frames (2001–2100), threshold, run the analysis, label the data, and it will save the results as a text file. It will then automatically repeat this for the next video sequence, and so forth. We can illustrate this using the brown trout experiment conducted by Purchase and Moreau (2012).

In the experiment there were two populations, 10 fish from each, five treatments, and five repeated microscope procedures for each of the above. This created 500 avi files, each of which was 41 s in length. In order to analyze motion from 10.0 to 10.5 s for each file, one would need to repeat the procedures in Table 1 500 times. To analyze motion at seven time periods (10, 15, 20, 25, 30, 35, 40 s) all procedures would have to be repeated 3500 times. That becomes very daunting and researchers therefore do not report such resolution. Our modified plugin does it all at once. Table 2 outlines the steps required to perform this analysis.

The two key pre-analysis steps are to decide on threshold values and plugin parameters ahead of time, and to appropriately name the video files. We use software called Prostream (http://prostream.southernvisionsystems.com/) to record video directly to the computer hard drive as it is inexpensive (approximately $150), but it will not record the file as an avi. Depending on the video acquisition software used one may be able to skip the following steps. In this experiment we recorded video at 200 frames s^{-1} for 41 s, producing three seamless Prostream files. For every 41 s of video from a microscope procedure we placed the files into a single folder (500 folders) under a unique name. For example, a folder named M_1_4_2 would mean population M, fish 1, pH 4, replicate 2; folder R_3_7_4 would be population R, fish 3, pH 7, replicate 4. In doing this we did not rename the files (all of the files have the same name to this point but are in different folders). After the experiment was finished, we converted all the Prostream files to avi format (Prostream will not record as avi, but will do the conversion from saved files), labelled the 500 avi files with their folder names (e.g. M_1_4_2.avi) and placed them into one folder. With great speed and ease these can be put through the automated procedures in Table 2. Everything is done at once, greatly reducing time required and eliminating risk of errors.

Time savings with the modified CASA_automated plugin

In the Purchase and Moreau (2012) example above, we used seven time periods from 41 s of data. Importantly, no extra work is required to extract data from every time period of useable data. For example, Fig. 1 shows the decrease in sperm swimming velocity with time post-activation for one fish at pH 6; the symbols are the average of five microscope

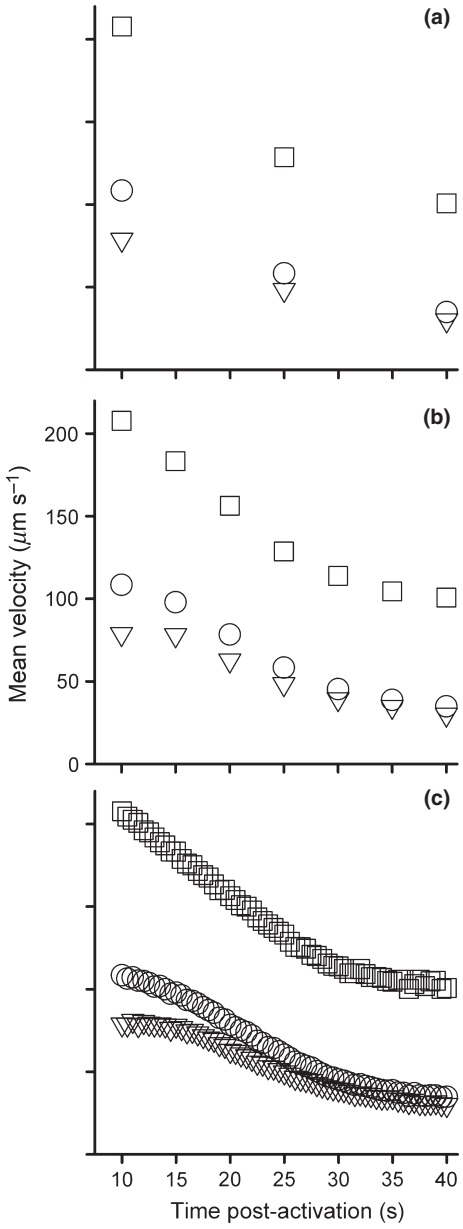

Fig. 1. Reductions in brown trout sperm swimming velocity as a function of time post-activation. Each line is the response of sperm from a single fish at pH 6. Squares are curvilinear velocity (VCL), circles velocity of the average path (VAP), triangles straight line velocity (VSL). Each datum is sperm movement over 0.5 s. Measurement frequency differs by (a) 15 s, (b) 5 s, (c) 0.5 s

procedures. In each case motion was quantified over 0.5 s. In panel (a), sperm motion was measured at 10, 25, 40 s post-activation. If this was performed with the original IMAGEJ plugin it would require 1500 separate computer analyses with our example experiment (500 microscope procedures for 20 fish and five treatments). At a conservative 3 min per video to do all of the steps in Table 1 this would take someone 75 h of desk time. In panel (b), sperm motion was quantified every 5 s as in Purchase and Moreau (2012) which would require 3500 separate analyses and labelling procedures, and is therefore never done to this resolution. In panel (c), the full power of the automation is evident as sperm movements have been quantified every 0.5 s, which would require 30 000 separate analyses without automation. Doing this with the original IMAGEJ CASA plugin would take 1500 h of desk time if only 3 min of total time is required for each video

sequence as in Table 1. Moreover, the potential that errors would be created in copying and labelling data outputs would be substantial. Our CASA_automated plugin can do this with a few computer mouse clicks.

We have tested the performance of the modified plugin with MS Windows and Linux systems but not with Mac OS (there should be no problem). The modified plugin, associated macro file, and a readme.txt file on its setup can be downloaded from www.ucs.mun.ca/~cfpurchase/CASA_automated-files.zip. A link to the first 21 s of each of the five video files used to create Fig. 1 and be found at http://philos.biol.mun.ca/Sample-videos-for-CASA_automated/.

Discussion

We have made changes to the open source IMAGEJ CASA plugin that greatly improve efficiency when working with large numbers of videos of swimming sperm. As a result, post-microscope computer processing time is appreciably reduced. This will allow researchers to conduct more complex experiments, using more animals and replicating microscope procedures more often, resulting in better science. We have made the new CASA_automated plugin freely available.

We want to highlight an unrelated issue with the IMAGEJ CASA software (and perhaps other CASA software). Users pick parameters to define objects in images as being sperm heads, and further define parameters to identify moving sperm from non-motile cells. From this researchers often extract data on percent motility. If sperm are tracked for a defined time period (e.g. 0.5 s at 200 frames s^{-1} would be 100 frames) and requirements are set that cells must threshold for all frames within that time period, then variation in sperm swimming speed will bias estimates of percent motility. This occurs because CASA will identify dots as sperm if they are non-motile or if they are moving and stay in focus for the total time period set (e.g. 100 frames). Faster moving sperm are less likely to stay in the field of view for the full time period than slower moving sperm, and thus a lower portion of fast moving sperm give complete tracks, and are therefore not counted. Hence, with the same microscope procedure, the count of the number of sperm present will increase as moving sperm slow down with time. This will bias changes in percent motility with time post-activation and would similarly bias this parameter between fish or treatments.

Acknowledgements

The ideas for the CASA improvements were developed during experiments that were funded by grants to CFP from the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, and the Research and Development Corporation of Newfoundland and Labrador. Laura Weir, Darek Moreau and Jason Lewis provided critical input into how to improve the functioning of the original IMAGEJ CASA plugin. The bias in identifying percent motility was discovered by Laura Weir while working in the laboratory of CFP. Comments from José B. Santos, and an anonymous reviewer improved an earlier version of the manuscript.

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