Computer assisted sperm analysis using ImageJ; description of necessary components and use of free software

Abstract:

Although the efficacy of computer assisted sperm analysis (CASA) has been demonstrated for use with a variety of species in assessing male reproductive quality as well as the impact of various treatments on sperm motility, many investigations continue to rely on manual analytical methods. CASA has been consistently shown to outperform such methods in terms of reproducibility as well as in the variety of parameters obtainable to describe sperm motility, however, its high cost has hindered widespread adoption. Also, that much of the information necessary to reproduce data obtained with commercial systems is proprietary information, and thus, not available, limits the ability to conduct comparative evaluations. Herein, we present a detailed manual for construction of a CASA systems based upon free software, which should increase the availability and utility of this technique.

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I. Construction of a video microscopy system

We have successfully performed CASA using video captured from a variety of microscopes and video cameras. Most commercial CASA systems utilize a 10x objective of a microscope; they do not, however, generally report the magnification factor used between the video camera and the microscope or the size of the chip in the video camera. In our experience, use of a 1x adapter in conjunction with a 10x objective and a 1/2" CCD or CMOS chip provides appropriate magnification. Although it is possible to use video captured from a brightfield microscope, the image produced by any of a variety of contrasting methods is preferable. (If brightfield microscopy is used, employment of contrast enhancing filters is almost a necessity and even with filters in place it may be difficult to distinguish between sperm and other particles). Phase contrast presents an image wherein the sperm head is bright; this results in high contrast and short shutter speeds. Darkfield can be used, but will require longer shutter speeds and dust on the slide or objective occasionally appears as bright as sperm, complicating analysis. Differential interference contrast does not appear to provide any advantage over phase contrast. Leica's Modulation Contrast may be more effective than general phase contrast due to the production of a very small bright center for each sperm; however, when sperm collide the bright portions do not touch and this may prevent the elimination of tracks due to collisions by our software. Zeiss' Varel Contrast does not appear to provide any advantage over phase contrast, and results in a less bright image.

In general, use of an inverted microscope may be desirable as focus will not be impacted with the application of sperm to the slide (this may decrease the time between application of sperm and acquisition of an image, a quality important in the analysis of fish sperm). Inexpensive microscopes with phase contrast can be acquired from Zeiss, Leica, Nikon or Olympus as well as Chinese manufactures such as Meiji and Motic instruments. The only characteristics we have found to be of importance in selection of a microscope are production of a bright image and the use of a planar objective. Resolution of the objective does not appear to impact analysis; we have had more success with the Zeiss A-Plan ph1 10x/0.25 than with the Plan-Neofluar ph1 10x/0.30 due to increased contrast. The objective used must be planar, otherwise portions of the field of view will always be out of focus; this will result in the elimination of sperm tracks by the software as sperm swim from one side of the field to the other. An electronic shutter speed shorter than 1/500th of a second appears to effectively freeze sperm motion; the camera sensitivity, microscope bulb wattage, condenser, and objective will impact shutter speed and thus should be considered when constructing a system. If the camera is coupled to the microscope by way of a trinocular tube, we recommend the prism directing light to the camera be 100/0 %, such that all light may be sent to the camera and none passed onto the eyepieces (this will increase brightness). It is possible to couple the camera through a binocular tube, but this will diminish brightness and if cost is an issue, use of a monocular tube would probably be preferable.

The most efficient flow of data is obtained by using a firewire machine vision camera and saving image sequences directly to a hard drive. A camera with the highest available frame rate should be employed as this provides the greatest amount of data and temporal resolution by locating the sperm at the most possible time points during analysis. Machine vision cameras operating at 100 frames per second may be best suited to the task as they are common and relatively inexpensive. Resolution of the camera does not appear to be of major importance and a camera with a low resolution 1/2" chip (659 x 490 pixels) is sufficient for recording the motion of 200 fish sperm using 10x total magnification. Possible sources for such cameras are Prosilica (prosilica.com), Basler (baslerweb.com) and Point Grey (ptgrey.com). Sources of software for writing image sequences directly to a hard drive include Unibrain (unibrain.com), National Instruments (ni.com) and the free software, Coriander (sourceforge.net/projects/coriander/). We have only performed data acquisition using a computer with a 3.4 ghz Pentium IV, 1 gigabyte of RAM, and two 7200 RPM SATA drives in a RAID 0 array. We were able to capture 100 frames per second at 659 x 490 pixels reliably for several minutes. The camera we used was a Prosilica CV640 monochrome 1/2" CMOS.

Alternatively, a consumer video camera could be mounted to a microscope via a specialized adapter, and the video processed using VirtualDub (virualdub.org); the video would either need to be converted to an uncompressed avi and opened in ImageJ with an avi plugin (http://rsb.info.nih.gov/ij/plugins/avi-reader.html) or saved as an image sequence. Importing video to ImageJ that is saved as an image sequence is accomplished by

way of the *File->Import->Image Sequence* menu. In this fashion one second of video from a specific time point in a longer recording can be opened.

II.   Processing video in ImageJ

As above, the video should be imported as an image sequence or uncompressed avi. This will open an image stack. If National instruments Labview is used to capture video it is possible to threshold video during acquisition; otherwise thresholding must be performed on saved video. This is accomplished in ImageJ using the *Image->Adjust->Threshold* file menu. Sliders should be adjusted so that sperm are selected (red) and the background is not. Clicking *Apply* will then discard all information other than the sperm heads, turning sperm into black circles on a white background. It may be difficult to produce video from sample to sample wherein sperm present the same contrast with the background (due to differences in focusing precision, primarily), as such, a different threshold setting may need to be applied to each video analyzed. Using a constant threshold at the time of acquisition alleviates this concern, as sperm will not be visible unless they are focused such that their brightness is great enough to be retained by the constant threshold value employed.

III. Analysis of video using CASA plugin

Use of this plugin depends on a basic understanding of ImageJ operation. We strongly suggest that individuals interested in using this plugin review the basic instructions for using ImageJ available at http://rsb.info.nih.gov/ij/docs/index.html prior to attempting to analyze sperm motility. Of particular importance is an understanding of the documentation regarding file menus, macros and plugins.

We have tested this plugin using multiple species of fish (unpublished observations) and fully explained our methodology (reference forthcoming). Analysis with this plugin goes quickly on our computer (specifications listed above), but we do not know if use of less capable hardware will result in an increase in the time required. We do recommend that the maximum amount of RAM available be alloted to ImageJ (*Edit->Options->Memory*). The parameters measured by this plugin are shown in the table below:

Characteristic	Description
Percent motility	Percent of sperm moving in a manner fitting motility determination parameters.
Velocity curvilinear (VCL)	Point to point velocity (total distance traveled) per second.
Velocity average path (VAP)	Point to point velocity on a path constructed using a roaming average. The number of points in the roaming average is $1/6^{th}$ of the frame rate of video used.
Velocity straight line (VSL)	Velocity measured using the first point and the average path and the point reached that is furthest from this origin during the measured time period.
Linearity (LIN)	VSL/VAP, describes path curvature.
Wobble (WOB)	VAP/VCL, describes side to side movement of the sperm head.
Progression (PROG)	The average distance of the sperm from its origin on the average path during all frames analyzed; division of this variable by VAP yields a description of the average efficiency in terms of the portion of generalized motion that results in movement away from the origin (a perfectly circular path will have a higher efficiency than an erratic path with the same linearity).
Beat cross frequency (BCF)	This value is determined in the plugin by detecting the frequency at which VCL crosses VAP. In cases where a sperm has a highly circular path, values generated are not valid (reference forthcoming). As such, we do not recommend use of this variable. Instead we suggest that directional changes per second would be a more valid measure. This measurement is present in the code of the program but is not output; we may substitute for BCF in the future depending on user demand.

The plugin must first be downloaded to the computer's plugins folder and compiled (*Plugins->Compile and Run*, locate the file and open, see http://rsb.info.nih.gov/ij/docs/menus/plugins.html for more detailed instruction). Running the plugin will result in initiation of a dialog box where parameters for analysis may be entered (and must be entered for each analysis performed - we recommend use of a macro to input these values, macros can then be saved with particular settings for a specific species). Macros can be recorded by opening the macro recorder *(Plugins->Macros->Record)*, and performing the analysis as normal.Clicking *Create* will open a new window with the text of the macro, running this macro will open the plugin, input the previously used values, and run the plugin.

See http://rsb.info.nih.gov/ij/docs/menus/plugins.html#macros and http://rsb.info.nih.gov/ij/developer/macro/macros.html#recorder for more detailed instructions regarding macro recording.

Output of the plugin will be the values mentioned in the above table, on a single row, separated by spaces (% mot VCL VAP VSL LIN WOB PROG BCF Number of sperm tracked). Multiple analyses may be preformed and will result in the addition of each analysis to a new row in the *Results* window. This may then be saved using the *File* menu on the *Results* window; save the file as "filename.csv". The file can be opened in a spreadsheet program by identifying the separator of values as a space (when opening, a dialog should appear asking how values are separated, one option should be *Space*). The plugin will also output an image the same size as a frame in the original video showing the paths of all analyzed sperm; grey sperm are ones that were identified as non motile. That all sperm were tracked can be verified by projecting the video; *Image->Stacks->Zproject*, choose *Min Intensity*. The projected image can then be compared with the tracks output by the plugin; cases where sperm were not tracked by the plugin generally occur because sperm move out of the field of view or out of the focal plane.

Values should be entered into the plugin's dialog box that accurately detect non motile sperm:

To be considered motile a sperm must meet the minimum VSL, VCL and VAP settings. It must also meet at least one of the following three sets of conditions (letters in quotes refer to the settings boxes in the screen capture below, refer to the values in this image for sample values that we have had success with):

1. WOB below high WOB "m", LIN below high LIN "n", maximum percent low VAP between frames "j" condition met, and maximum percent zero VAP between frames "i" condition met (this set of conditions is intended to find sperm that are in motion for most of the time analyzed and that do not move in a perfectly straight line as would be expected of sperm moving due to bulk flow).
2. VCL above low VCL "l" and VAP above low VAP 2 "k" (this set of conditions identifies sperm moving faster than is characteristic of bulk flow regardless of their motion characteristics)
3. WOB below high WOB two "o", and LIN below high LIN two "p" (this set of conditions identifies slow moving sperm with a high degree of path curvature as such curvature is not characteristic of sperm moving due to bulk flow)

Correct settings should be determined experimentally for the species of interest. The primary purpose of using two passes in detecting motile sperm is to increase the plugin's ability to identify slow sperm as motile, while not incorrectly classifying sperm moving due to bulk flow of the sperm suspension. Analysis of mammalian sperm, which can be recorded minutes after application of sperm to the slide (and thus after most bulk flow of the fluid has ceased) might be performed by applying generally reported velocity specifications (such as a VCL above 25 µm/s) in the first pass "g" and negating one of the second pass controls (by, for instance, setting high WOB two "o" and high LIN two "p" to 200, such that any sperm qualifying for the first pass setting will also meet these second pass conditions).
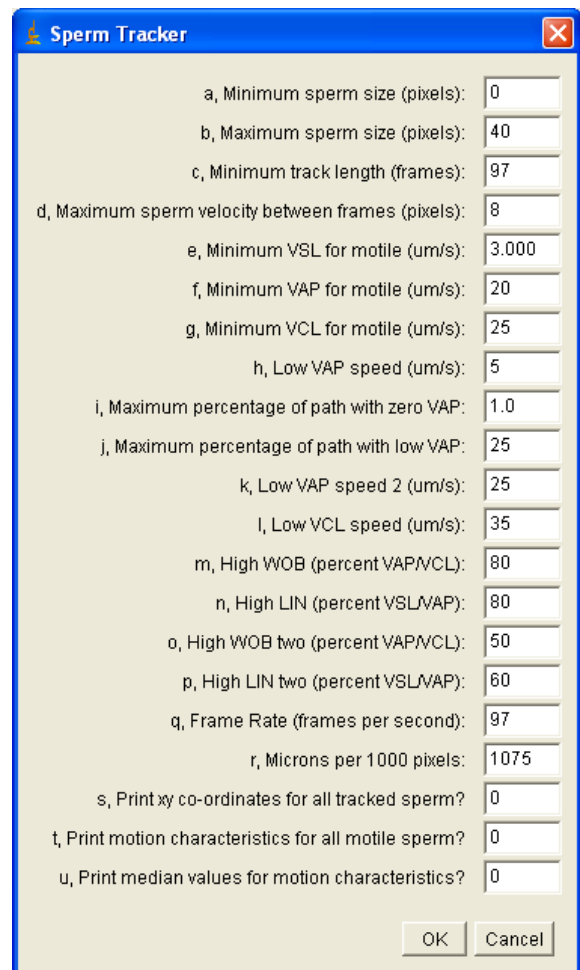
Dialog box generated by CASA plugin within ImageJ, values may be entered into fields in order to determine data output and to input parameters for sperm path construction and motility determination. Values shown in the fields represent those that we have used to successfully analyze fish sperm motility.

Format of the plugin's output:

When the number "1" is entered in the *Print all motile sperm motion parameters field* "t" the following data will be output for each sperm (entering "0" in this field with prevent this data from being output):
% mot, VCL, VAP, VSL, LIN, WOB, PROG, BCF

When "1" is entered in the *Print x,y co-ordinates for all sperm field* "s", x,y co-ordinates separated by a space will be printed for all sperm analyzed (motile and non-motile), entering a "0" in this field prevents these data from being output. We suggest using this for individual sperm only, as formatting is not applied to distinguish the co-ordinates of one sperm from the next (analysis of a single sperm can be completed by clearing the regions of the image containing other sperm – this is accomplished using selection tools and the *Edit->Clear* function). The first set of co-ordinates in each row represents the centroid positions of the sperm in all frames, the second set represents the VAP co-ordinates (fewer are printed because this can not be determined until a point in the sperm path where enough co-ordinates are present to average), and the third set of co-ordinates in each row represents those data used to determine VSL.

At the end of the list of individual sperm motion parameters the average values for the population of sperm will be output in the same format (if individual sperm parameters are not printed, then this is all that the plugin will output into the *Results* window), additionally, the number of sperm analyzed will be written after BCF. If "1" is entered in the field for *Print median motion characteristics* "u", the median values for the sperm population will be output between the average BCF and the number of sperm analyzed (entering the value "0" will prevent this data from being output), and the complete data format will be as follows:

% mot | VCL | VAP | VSL | LIN | WOB | PROG | BCF | Median VCL | Median VAP | Median LIN | Median WOB | Median PROG | Median BCF | Number of sperm tracked

Please contact the authors for assistance or clarification or refer to (reference forthcoming).