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3. **Dates of visit:** 06/02/12-08/14/12
4. **Title of protocol:** How to use ImageJ with CASA plugin (Computer Assisted Sperm Analyzer)
5. **Rationale and background:** This protocol provides a detailed description (along with parameters) on how to use a free and objective method to analyze sperm movement which is easier and more efficient than traditional manual analysis. This protocol is based on videos captured with Zeiss Axiovision (.zvi files) but could be easily modified for other file formats.
6. **Protocol:**

Read over: <http://rsbweb.nih.gov/ij/plugins/casa.html> and especially <http://rsb.info.nih.gov/ij/plugins/docs/CASAIstructions.pdf>

Materials:

- Computer
- External hard drive

Setup

1. Download ImageJ, a free image processing and analysis software. (<http://rsbweb.nih.gov/ij/>)
2. Install CASA plugin. (http://rsb.info.nih.gov/ij/plugins/download/CASA_.java)
3. Right click the page, click Save Page As., save the CASA java code into the plugins folder within the ImageJ installation. (My Computer > Local Disk > Program Files > ImageJ > plugins)
4. Install Mosaic plugin. (<http://www.mosaic.ethz.ch/Downloads/ParticleTracker/>)
5. Click on "[as ImageJ JAR file](#)" and save into the plugins folder within the ImageJ installation. (My Computer > Local Disk > Program Files > ImageJ > plugins)

Video export from Zeiss AxioVision

(It is a good idea to export videos onto an external hard drive.)

1. Open Zeiss AxioVision to export videos previously captured in bright field and saved as .zvi files
2. File > Open Image. (or Ctrl + I)
3. Open the video to be analyzed. (Videos with high contrast between sperm heads and the background work best. Try to avoid videos with inconsistent backgrounds.)
4. Create a folder specifically for the opened video on an external hard drive. (e.g. ###)
5. File > Export. (Or Ctrl + F6)
6. Click on the "... " button to the right of the "Save in:" box.
7. Open the folder you just created.
8. Make sure "Generate merged image(s)" is unchecked and "Convert to 8 bit" is checked.

9. Click Start.
10. Repeat steps 2-8 until all videos have been successfully exported.

Record CASA macro

1. Open ImageJ.
2. Plugins > Macros > Record.

Copy and paste the following (without quotations) into the blank Macro. (*Preferred method*)

```
run("8-bit");
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");
run("Invert", "stack");
run("Background Subtractor", "length=20 stack");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(100, 255);
run("Convert to Mask", " ");
run("CASA ", "a,=1 b,=250 c,=20 d,=20 e,=3.000 f,=20.000000000 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,=(INSERT FRAMES PER
SECOND FOR EACH VIDEO HERE) r,=1000 s,=0 t,=1");"
```

Note: The q value represents the frames per second (FPS) of the video, which may change between different videos depending on how the videos were taken.

3. Change the Name to "CASA.ijm".
4. Click "Create".
5. Save the newly created Macro within the "macros" folder in the ImageJ installation. (File > Save As)

To record your own Macro,

1. Open a random video. (File > Import > Image Sequence)
2. Plugins > Macros > Record. (ImageJ will now record all of your clicks into the macro so follow the next steps carefully)
3. Change the Name to "CASA.ijm".
4. Image > Type > 8-bit. (This will convert the all images into 8-bit format, which is required for CASA.)
5. Analyze > Set Scale > Click to Remove Scale > OK. (This will remove the scaling on all images, which is required for CASA.)
6. Edit > Invert > OK. (This will invert the color on all images, which is required for CASA.)
7. Plugins > Mosaic > Background Subtractor > OK. (This will determine the background and darken it to provide greater contrast from sperm heads.)
8. Image > Adjust > Threshold.

9. Drag the first slider to “100” and leave the second one at “255”. (This may need to be determined experimentally depending on how much contrast there is between sperm and the background, but it should be left constant once an appropriate value is determined.)
10. Make sure “Dark background” is checked.
11. Click on “Apply” and then “OK”. (This will remove the background leaving only black dots that represent sperm heads.)
12. Plugins > CASA.
13. Use the following settings:
 - a. 1
 - b. 250
 - c. 20
 - d. 20
 - e. 3
 - f. 20
 - g. 25
 - h. 5
 - i. 1
 - j. 25
 - k. 25
 - l. 35
 - m. 80
 - n. 80
 - o. 50
 - p. 60
 - q. **FPS** (You can calculate this by taking the # of frames and dividing by the # of seconds the video was taken. This value may change between different videos).
 - r. 1000
 - s. 0
 - t. 1

Note: For the identity of CASA parameters, look here:

(<http://rsb.info.nih.gov/ij/plugins/docs/CASAIinstructions.pdf>)

These values may need to be determined experimentally as well, but should be left constant once appropriate values have been determined. Parameters a, b, c, d, are easiest to adjust.

14. Click “OK”.
15. Save the Macro you’ve created within the “macros” folder in the ImageJ installation.
(File > Save As)

Video analysis

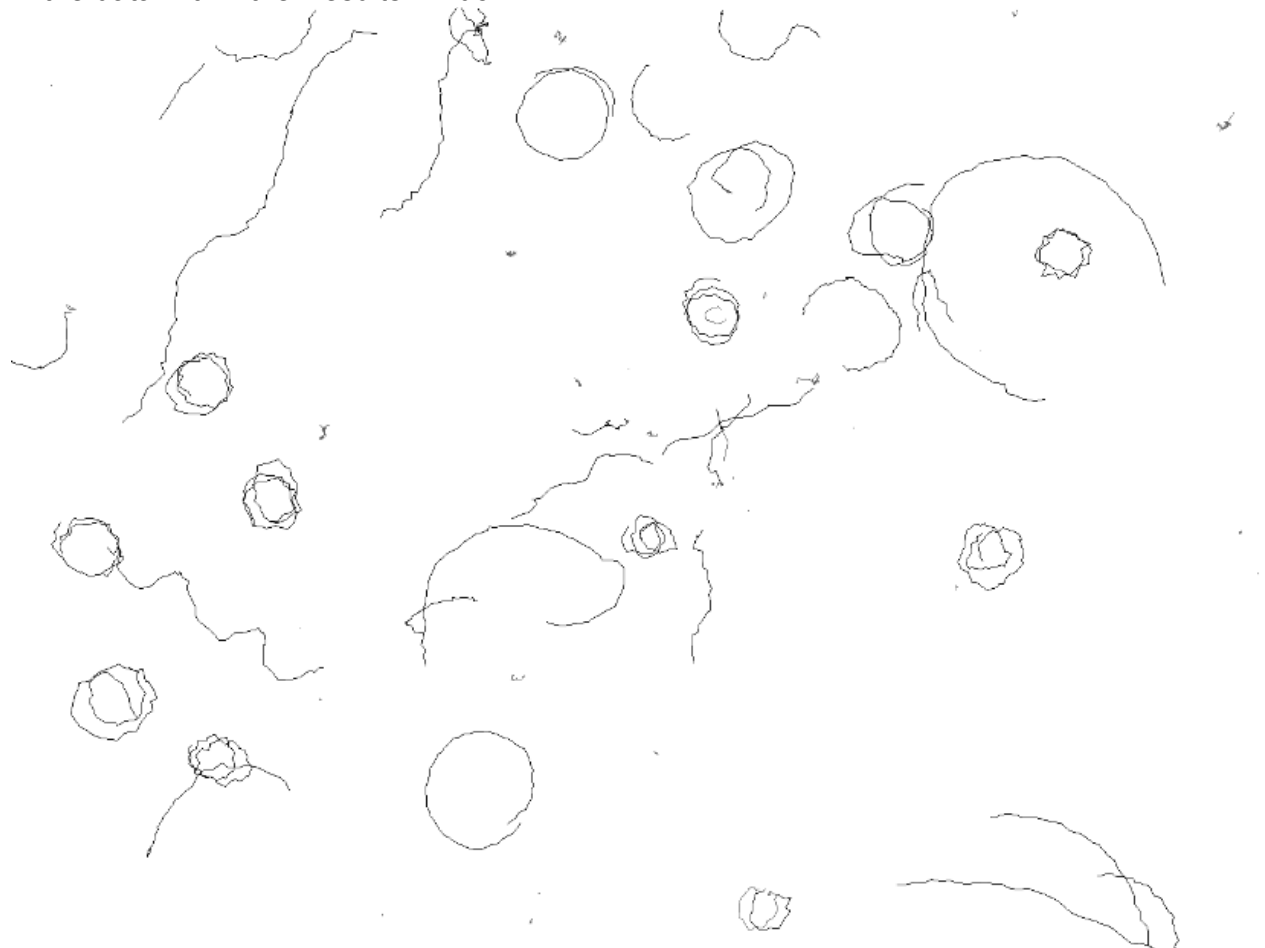
1. File > Import > Image Sequence.
2. Open the first image for the image sequence about to be analyzed.

3. If you do not know frames per second (FPS) of the video, you will need to remember the “Number of images” to calculate it.
4. Click “OK”.
5. Plugins > Macros > Edit.
6. Find and open the macro you created. (“CASA.ijm” within the macros folder)
7. Change the “q” value to the correct FPS for the video you are analyzing. (# of frames / # of seconds)
8. Run the macro by clicking Macros > Run Macro. (or Ctrl + R)

Three windows will now pop open.

The **Results window** shows a list of all output data for tracked sperm in the following format:
VCL | VAP | VSL | LIN | WOB | PROG | BCF | Number of sperm tracked
The final line displays the average values including the % motile in the first column.

The **Paths window** shows track lines for each sperm cell. Black track lines represent motile sperm while grey track lines represent sperm that were not motile and were not represented in the data within the Results window.



The **Modified Sperm Video window** shows the sperm video after processing. Only black

dots representing sperm heads should be visible.

9. Select all the data in the Results window. (Ctrl + A)
10. Copy (Ctrl + C) and then paste into an Excel sheet. (Ctrl + P)
11. Delete all the data within the Results window.
12. Close the Results window.
13. Bring up the Paths window.
14. Bring up the ImageJ window.
15. File > Save As > Tiff.
16. Rename (###_paths) and save into external hard drive.
17. Close the Paths window.
18. Bring up modified sperm video window.
19. Bring up the ImageJ window.
20. File > Save As > Tiff.
21. Rename (###_final) and save into external hard drive.
22. Close the modified sperm video window.
23. Repeat steps 1-22 until all videos have been analyzed.