# Sperm analysis

# Sperm cell measurements on the Leica microscope using LAS 4.1

## Preamble

Morphometric measurements of sperm cell components (head, midpiece and tail) has traditionally been performed on the Leica microscope currently located in room ZM045, using the associated software Leica Application Suite (LAS). The current version of LAS is 4.1. This procedure describes how to perform such measurements in compliance with how previous measurments have been made.

The settings will have to be set whenever a new user starts to use the system, but will subsequebtly be associated with the user. The two first settings sections can therefore normally be skipped.

## Preparations

1. Turn on the microscope
2. Log on to the computer. All settings will normally be set to the same state as last time one was logged on, but sometimes e.g. brightness will have to be adjusted
3. Start the Leica Application Suite software (LAS 4.1)

## Settings

### Microscope settings

Use the greenish display at the microscope to enter the following settings.

#### Light settings

* Tap on Light settings symbol (dark and light half circles) in the left part of the display
* Tap on BF to select Brightfield

#### Magnification

A magnification of 160X, or 320X for species with short sperm cells, will normally be suitable for sperm morphometric measurements. Higher magnification makes it easier to measure the single sperm cell, but will require more images to be taken in order to include enough sperm cells on the images. To set the magnification to 160X;

* Tap on the Magnification symbol (nosepiece & magnifying glass) in the left part of the display
* Tap on the value 10 to select the 10X nosepiece
* Turn the metal wheel at the upper right side of the microscope body so that the value 1.6x is shown
* The microscope display should then indicate “Σ 160 x” in it’s lower part

### Software settings

#### Setup tab – Nosepiece – Fine tuning

Here, under Objective Parameters (lower panel), the stepsize for focusing may be set for each objective separately. Select the objective of interest and choose a level in the Stage and Z-Stepsize drop-down box; S1 will normally be a good choice.

#### Aquire tab – Mic1

Adjust the Aperture (Ap.), Field (Fld.) and Light intensity (Int. Fine) settings so as to achieve a best possible image. Try to keep the Aperture as low as possible without the image getting blurry, and then adjust Light intensity until the image looks OK. The following settings should be a good starting point for 10X and 20X nosepieces, respectively:

 **10X 20X**

Apperture 8 12

Field 17 12

Int. fine 98 116

These values fit well with the Exposure time (found under the Camera tab; see below) set to ~ 40 ms.

#### Aquire tab – Mic2

Nothing needs to be changed here, but this tab may be useful for fine tuning of focus (easy to see how much the focus is shifted). Here you may also shift between Fine and Coarse steps for the focus adjustment, and between Precise and Fast movements of the slide table.

#### Aquire tab – Camera

Make sure that none of the symbols in the first panel, DFC 420 – Last used, are selected (they should have a black, not orange, background colour). The panels mentioned below should be visited to make sure that their settings are correct; for the rest of the panels all settings can be left unchanged (i.e. set to Off or None).

**Exposure Adjust**

Exposure 39,2 ms

Gain 1,0 x

Saturation 1,50

Gamma 0,60

**Input options**

Store your own configuration under Configuration (alternatively use one of the “LarsErik…” configurations with the latest date), so that you can be sure to always use the same settings:

* Click on the button with a + on and provide a suitable name for the configuration

**Image formats**

Captured format 2592 x 1944, Interlaced Large HQ

* 1.5x scaling No

Live format 864 x 648 Progressive Large OR 2592 x 1944 Interlaced Large

Image type Colour

864 x 648 Progressive Large is generally sufficient, and fast, but for better on-screen resolution 2592 x 1944 Interlaced Large may be chosen (but will be slower).

**Calibration settings**

Type Calculated

Configuration Default

Actual length of line shown on image 200 Microns (if it can be changed; otherwise leave as is)

### Preferences

Choose Preferences from the Options menu at the very top of the window (or hit Ctrl+O) and check the following settings in the following tabs (all other settings should be left unchanged):

#### Defaults

Default Action Acquire

Image table field for description Description

Default Action When Image Rejected Show image

* Display dialog Yes

#### Image

Always Confirm Image Name No

Capture to fixed folder location Yes

Always create thumbnail file Yes

Default Image Name To be set for every new individual sample (see below)

Leading zeros 2

In this format Jpeg (Best Quality)

DPI 300

After Capture Open in –> Analysis

Decimal places 2

## Setting capture location (where to save the images)

1. Go to the Browse tab
2. Locate your folder in the Folders tree at the left side of the screen
	* Files should be saved to a folder with your name on the local D drive, so that they may be available also for others in the future
3. Click on the third button from the left in the Navigator pane (Set capture location) to save images to that folder

## Taking an image

1. Enter individual ID info under Options – Preferences (Ctrl+O) – Image in the field Default Image Name, using the following pattern;
* Six-letter species acronym (e.g. CyaTen)
* Underscore
* Corema accession number (only numeric part, e.g. 12345)
* Underscore
* Corema item number (only numeric part , e.g. 2)
* Underscore

Example: *CyaTen\_12345\_2\_*

LAS will automatically add a running number for subsequent images taken of the same sample (i.e. with identical Default Image Name), and the resulting image name of the two first images taken will then be:

* *CyaTen\_12345\_2\_.jpg*
* *CyaTen\_12345\_2\_01.jpg*

Since LAS 4.1 does not add any empty space before the running number, a trailing underscore should be included in the Default Image Name so that the item number can be separated unequivocally from the image number.

1. Copy the entered Default Image Name, except for the last underscore, to store this in the clipboard for later use
2. Go to the Acquire tab and make sure that the settings are OK, e.g. by choosing the previously saved profile under Input options in the Camera tab and adjusting Aperture, Field and Light intensity if necessary
3. Locate the sperm cell(s) to be imaged (see Sperm cell selection criteria below). In order to set the focus plane correctly, it may be smart to start on 10X magnification, position the slide so that some of the text on writing field is in center and then focus until the text gets focussed, before trying to locate actual sperm cells.
4. Find a blank area near the sperm cells (no objects, just background), draw a square by left-clicking and dragging, and choose White Balance from the popup menu to set the white balance
5. Focus and hit F3 to capture the image. Check the resulting image, which will open in the Analysis tab. If the focus is not perfect, go back to the Acquire – Mic2 tab, adjust the focus in small steps (2-3 µm at the time) and take new images until the best possible focus is obtained.
6. In Analysis the image can be zoomed after having clicked on it once in the viewer. To move around, the Pan Window (button with a hand in the toolbars at the right hand side of the screen) may be useful. Also the Shift-key is useful; hold it down to zoom in on the area around the pointer.
7. To start measuring, in Analysis choose the SegmentLine Tool (the third tool from the upper left in the Measure Tools - Selection box). Check under Properties that Line Thickness and End Bar are set to 1, and that nothing has been ticked off under Display Labels (this has to be done every time the software is restarted).
8. Zoom in on the head of the sperm cell and locate the exact endpoint of this (see Sperm cell selection criteria below) by holding down the Shift-key.
9. Left-click once at the start (front) of the head to start measuring; left-click once (or more) again to change direction of the measurement line; finally left-click at the place to end the measurement line and then right-click to end the measurement. Repeat this for midpiece and tail, and repeat for all three component for each new sperm cell to be measured.
10. After all measurements have been done on an image, check under Select results that the relevant measures are included in the output. When using the system for the first time a grid layout should be saved under Current Grid Layout, with the current settings:
	* Select Grid: Details
	* Configure: Choose the following items to be visible;
		1. Measurement #
		2. Class
		3. Image Name
		4. Line Length
11. To save the data, go to Create Report, fill in sample info and make sure the the other settings are as follows:
	* Fields:
		1. **Specimen:** Sample info (e.g. *CyaTen\_12345\_2*). By clicking Ctrl+V this can be pasted in, given that it was copied into the clipboard in 8 above
		2. **Description:** Sperm
		3. **Observations:** Your initials
	* Output:
		1. **Format:** Excel
		2. **Append:** Yes
		3. **Attach to Record:** No
	* Content:
		1. **Details:** Yes
		2. **Original Image:** No
		3. **Result Image:** No
	* Click Export and choose an appropriate file location and name (again, the Default Image Name can be pasted from the clipboard). Click Export once again to actually export the data
12. If multiple images have been measured for the same ind., repeating step 18 for each image will append the measurements from each image to the same Excel file. If you choose to open the report after each export (by ticking off for Display Report in the Export dialog box), this will have to be closed again before new data can be added to the file. Normally this setting can be kept off until data from the last image for an individual is saved.
13. Access images (e.g. images not used to measurem sperm cells on due to bad focus etc.) may well be deleted. This should then be done in the Browse tab; select the image(s) to be deleted and hit Delete on the keyboard.

## Sperm cell selection criteria and how to measure

### Selection criteria

Only normal, intact sperm cells, for which all elements crucial for the measurements can be seen (see below), should be used for morphometric measurements. It may sometimes be difficult to decide whether a sperm cell is normal and intact, but after having gained some experience with the sperm cells of a given species one will normally be able to identify suitable cells for measurements. The following criteria should generally be adhered to:

* All parts of the cell should be present and intact; pay special attention to:
* Head: make sure that the tip (acrosome) is not broken of or malformed, and that the head is not broken or abnormally shaped
* Midpiece: the membrane winding around the midpiece may in some cases have started to disintegrate towards the rear (tail) end. It may then protrude from the midpiece or seem to have become detached from the “central chord” of the midpiece. Such cells should preferrably not be used for measurements
* Tail: the tail should “fade out” towards the tip (i.e. become progressively thinner); an abrupt end to the tail is likely to be an indication that it is broken, and the cell should then not be used for measurements
* All measurement points must be clearly identifiable. These include:
* Tip (foremost point) of head
* Transition Head-Midpiece
* Transition Midpiece-Tail
* Tip of tail

### How to measure

#### Head

* Start the measurement at the foremost pixel that can be identified as belonging to the head (see Fig. 1)
* Be aware that the cells often will be surrounded by a lighter “halo” which should not be included in the measurment
* Follow the center line of the head, disregarding any undulations caused by the spiral form of the head. However, if the head is bent or lying in a curved shape, the measurement line should also be bent in order to reflect this.
* End the measurement at the rear end of the lighter area forming the center of the head, i.e. at the center of the transition from lighter (whitish) to darker (brownish) colour (see Fig. 1).

#### Midpiece

* The measurement should ideally be started at the exact same pixel as where the head measurement ended, but the LAS software does not allow this (!). Therefore, try to move the pointer a pixel or two sideways (perpendicular to that point) to start the midpiece measurement (lenghtwise overlap of the two measurement lines should be avoided to ensure that the total sperm length is not over-estimated).
* Follow the center line and any curvature of the midpiece; the measurement line should always stay “within” the midpiece sideways (e.g. it should not cut outside the midpiece in an inner curve)
* End the measurement where the membrane undulating around the midpiece (and thus the midpece) ends (see Fig. 2). This transition may be difficult to identify, but the following tips may be useful;
	+ Looking at several images with slightly different focus planes, as the transition may be more evident even on a slightly out-of-focus image
	+ The midpiece will often have a repeating pattern of darker and lighter spots and a slightly undulating outline, both resulting from the spiral-wound membrane
	+ The tail will normally look plain and uniformely coloured, and with a straight outline

#### Tail

* Start the measurement where the midpiece measurement ended (given the same limitation as described for midpiece above)
* Follow the center line and any curvature of the tail, as described for the midpiece
* End the measurement at the rearmost pixel that can be identified as belonging to the tail (see Fig. 3). It may often be useful to squint or peer at the screen in order to identify this point.

## Export and import of images & associated meaurements files

See separate Best Practice Manual.