SCA® Quick User's Guide for Human 6.5   English Version

SCA® - Sperm Class Analyzer®
SCA® Quick User's Guide for Human

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CE

MICROPTIC
AUTOMATIC DIAGNOSTIC SYSTEMS

EC – DECLARATION OF CONFORMITY

We herewith declare that the following In Vitro Diagnostic Medical device meet the transposition into national law Royal Decree 1662/2000, the provisions of Council Directive 98/79/EC of 27 October 1998 In vitro diagnostic directive. All supporting documentation is retained at the premises of the manufacturer.

Manufacturer:
MICROPTIC S.L.
Av. Josep Tarradellas, 8, 1º 6ª
08029 - Barcelona (Spain)

Generic Name: **Sperm Analyzer**

Product Name: Sperm Class Analyzer - SCA (CASA system)

Classification: Not listed A or B, Article 9 – (Self declare)

Directives:
In vitro diagnostic Directive
(Annex III - Self declare) 98/79/EC

Classification: Other General Device

Date: 30/03/2020

Standards:
ISO 9001:2015   (Quality Management)
ISO 13485:2016 (Design, Production, Installation and Servicing)
EN 62304:2006/A1:2015 (Medical Device Software)
EN 62304:2006/AC2008 (Medical Device Software, update)
EN ISO 15223-1:2016   (MD symbols)
EN ISO 18113-3:2009   (IVD labelling)
EN 61010-2-101:2017   (Safety for IVD)
EN ISO 14971:2012   (Risk Management to Medical Devices)
EN 62366:2008   (Usability)

Responsible person, position in company:

Eduard Sánchez Mallol, CEO

Signature:

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CASA
SPERM CLASS ANALYZER

INTENDED USE

SCA is software for capturing images through a digital camera installed in a microscope, aiding in the assessment of human semen samples, following the WHO guidelines.

1 SCA® Sample Management

1.1 Executing the application

*1. To start the application, click on the **SCA® evolution** icon displayed on the desktop.*

*2. The system comes with a user account called **Administrator** that doesn't need any password. To login into the system just select **Administrator** user and press OK.*

1.2 Customize the application

1.2.1 Create new users and roles

*1. It is possible to create new users and assign a role (user profile) to each one. To see the different roles go to menu **Administration > Roles**.*

*2. By default the system comes with three Roles (**Analyst**, **Administrator** and **Viewer**). You can add more roles or change the permissions (full access, read-only or no access).*
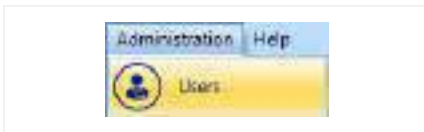
*3. To insert new Users go to the menu **Administration > Users**.*

*4. Press the **Add** button to add new users or select a user from the table to modify their roles, name or password.*

1.2.2 Modify/add labels

It is possible to modify all of the labels in the SCA program (words used on the program) and also add new description to the fields describing the sample data (options available for a field).

1. Change any word of the program by going to the menu **Administration > Labels** and a window with two columns will be displayed.

2. The left column contains the default text of the labels, the right column contains the customized text of the labels that will be shown and can be modified with the preferred terms**.**

Another way to modify a word is with a double click on the text at any window of the program or with right click on the text and selecting the "Change word" option.

3. Change/add words to the options in the fields of the macro/microscopic data by clicking on the menu **Settings** you will see the list of the fields shown in the main screen.

4. Select the specific term you want to modify and a window will open that allows you to add new terms (for example if you click "Appearance" you will see that by default there are only "Normal" and "Abnormal" you can add for example, "Reddish", "Yellow", "White"). The existing options can also be modified.

Another way to manage the options of a field is with a right click on the text of the field (in the macro/microscopic data) and selecting the "Edit items" option.

1.3   Create a new sample

When you run the application, the default is to begin a new analysis. The reference number will always be the following one from the last sample reference number (you can alter this if you want and use a new one).

*1. To select an existing patient: Insert the **CHN** (Clinical History Number) and press the **Search** button (magnification glass button).*

*2. If the patient doesn't exist in the database, press the **Add Patient** button and write any details about the patient. The **CHN** and **First Name** is obligatory, all other fields are optional.*

According to the WHO manual for the Examination and processing of human semen, 5th Edition: "Semen analysis should begin with a simple inspection soon after liquefaction, preferably at 30 minutes, but no longer than 1 hour after ejaculation to prevent dehydratation or changes in temperature from affecting semen quality."

3. Fill all other fields of the macro/microscopic data window.

* The volume is best measured by weighing the sample in the vessel in which it is collected.
- Collect the sample in a pre-weighed, clean, disposable container
- Weigh the vessel with semen in it
- Substract the weight of the container
- Calculate the volume from the sample weight, assuming the density of semen to be 1g/ml.

* WHO laboratory manual for the examination and processing of human semen, 5th Edition.
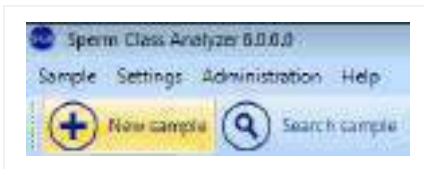
4. If you want to create more new samples, press the **New sample** button. A new tab will be added on the top of the window.

1.3.1 Panel of recently added samples
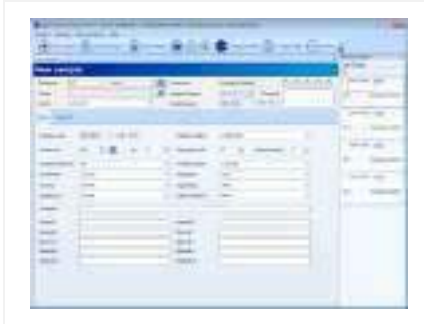
1. On the right side of the screen the recently added samples are displayed. This panel is offering quick access to these samples by clicking on the reference number in case the user wants to recall a recent analysis.

1.3.2 Searching a sample

*1. You can search any existing sample with the option **Search sample** in the Sample Management screen.*

Searching can be done by inserting the following criteria:

- CHN of the patient (Clinical History Number) or sample reference.
- Patient Name and Surname.
- Analysis date.
- Analyst.
- Centre.

*2. Press the **Search** button to see all the analysis carried out on a selected sample. You may sort the results of the analysis by pressing the header of the column.*

2   SCA® Analysis Programs

*1. Once the patient and sample data has been entered click on the button **SCA® Programs** and select the program you want to open. You can also enter the programs by clicking the icons from the results tab.*

Analysis programs available:

Automatic analysis programs:

- ***SCA® Motility and concentration***
- ***SCA® Morphology***
- ***SCA® DNA Fragmentation***
- ***SCA® Vitality***
- ***SCA® Acrosome reaction***
- ***SCA® Leukocytes***

Manual analysis programs:

- ***SCA® Manual counter***

2.1 SCA® Motility and concentration

2.1.1 Protocol for motility and concentration evaluation

Protocol for motility and concentration analysis:
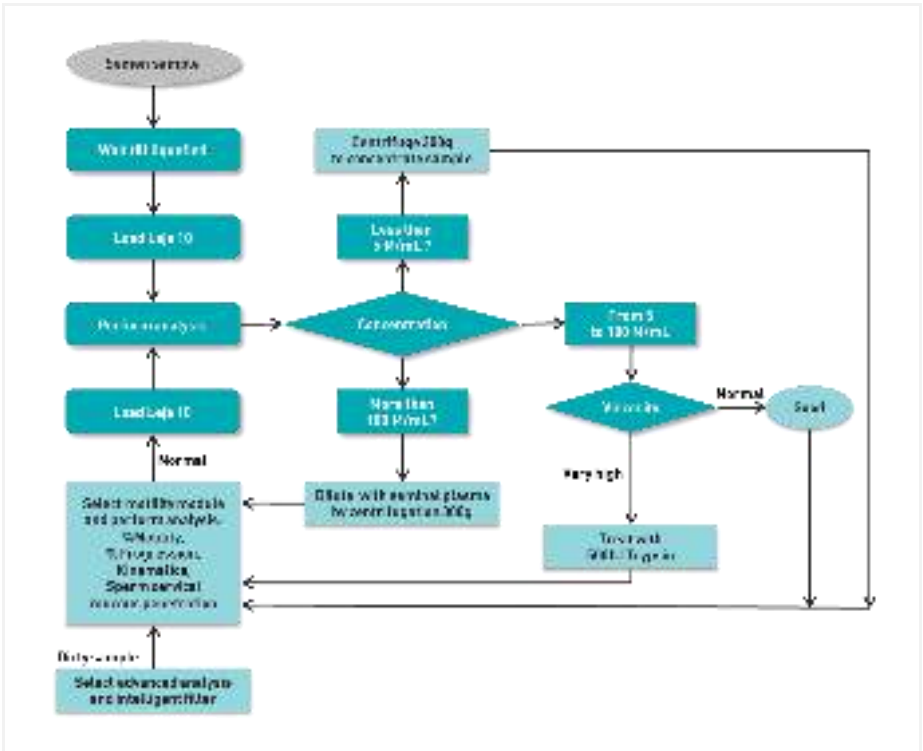
More information about sample preparation:

Follow all sample preparation advises of the **WHO laboratory manual for the examination and processing of human semen**, World Health Organization, 5th Edition (2010).

At the end of this manual you can also find a **Manual for sample preparation** (Appendix 1 and 2).

2.1.2 Steps to follow

*1. Microscope: Insert the **green filter**.*

*2. Condenser: Select the **phase contrast (Ph1)**.*

3. Place the specimen (using Leja slide or Makler chamber) on the microscope stage.

*Objective: Use the **10x positive phase contrast objective**.*
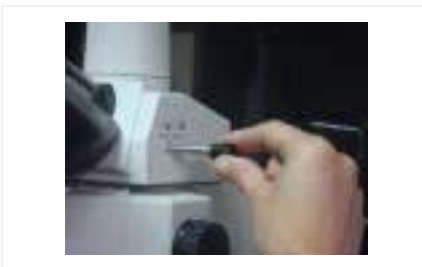
*4. **Focus the image** in the microscope and then **direct the image to the camera** in order to see the image on the computer screen.*

*5. Open the **SCA® Motility** module clicking on Programs button and then on SCA® Motility.*

*6. Press **Enter** in the configuration window.*

7. Check the current configuration (menu "Configuration" > "View/Edit").

8. Observe the main analysis properties:

- *Calibration: 10x*
- *Capture method: Ph+ (positive phase contrast)*
- *Disposable: Leja, Makler, etc.*

And press the "Save as default" button.

9. In main window, you will find information about the sample volume and dilution. Write the dilution if there has been any, if not leave at "0".

For the option "Post-treatment" consult the SCA® manual.

*10. Press the **Analyze** button in order to display a live image on the screen.*
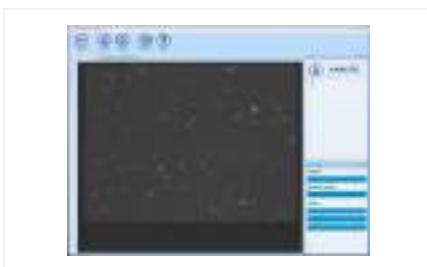
11. Focus the image on the screen and reach the best level of contrast (adjust microscope illumination in order to achieve a homogeneous background similar to the background colour of the window).

*Then capture the fields required by clicking the **Analyze** button (the software will advise you when enough fields have been captured).*
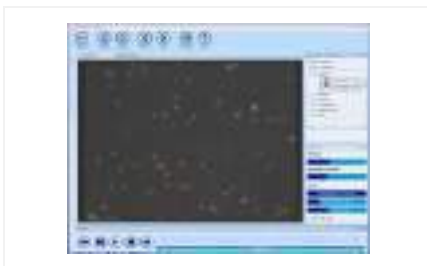
*12. After capturing a sufficient number of fields click the **Results** button.*

13. The main window will show the general results.

Analysis window main applications:
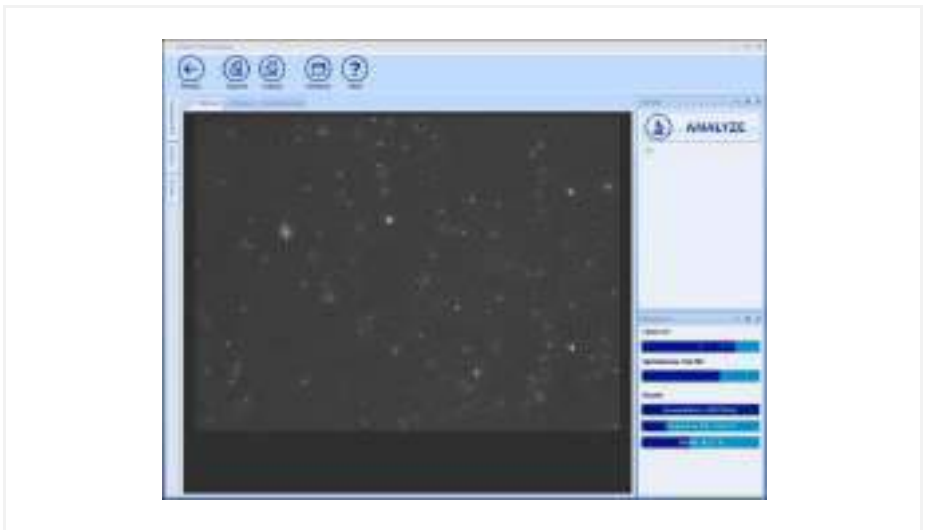
- Analyze: Click to start the analysis.

- Capture: View live image (start the capture).

- Analysis: View the last captured field (analysis).

- Results: Click to go back to the general results screen.

- Windows: Show/hide other panels in this window.

Note: For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)

Results window:

1. W.H.O. classification

2. Classification based on velocity and sort groups

3. Kinematic values

4. Fields comparison

5. Spermatozoa details

6. Data summary tool

Reports:

1. Open the Report by clicking the **Report** button. It is also possible to create reports from the Sample Management: Close the analysis program, save the analysis into the database and click the Reports option from the Sample management.

Report example:

2.2 SCA® Morphology

2.2.1 Protocol for Morphology evaluation

Protocol for morphology analysis:
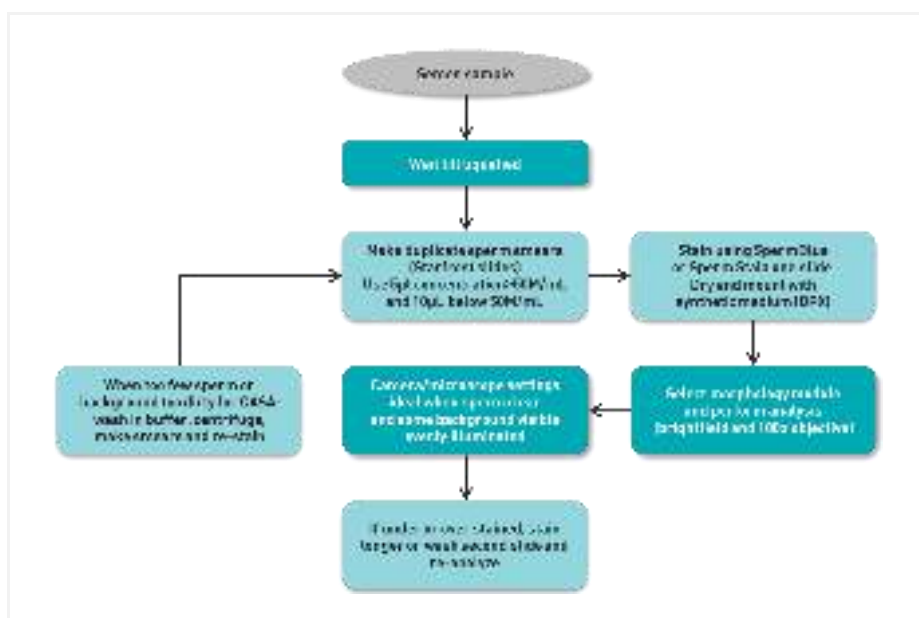
More information about sample preparation:

We recommend you to follow all the steps for sample preparation explained in the book *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction*, from World Health Organization, 5th Edition (2010).

At the end of these manual you will also find an Appendix for sample preparation.

The Number of fields / spermatozoa

According to WHO, the morphologic evaluation of the human sperm requires the analysis of minimum 200 cells twice, nevertheless, considering the quantity of and the quality of variables analysed with the SCA® it is enough to analyse **100 spermatozoa per sample**.

There are also differences in the sample smears, therefore it is recommendable to make **two smears of the same sample** and capture 50/100 spermatozoa in each.

2.2.2 Steps to follow

*1. Microscope: Insert the **blue filter**.*

*2. Condenser: Select the **brightfield position** (A or O) depending on the microscope.*

3. Place the specimen (fixed and stained with Sperm Stain, Diff-Quik or SpermBlue) onto the microscope stage.

Objective:

- *Standard camera: Put one drop of oil on the slide and use the **100x oil immersion objective**.*
- *High resolution camera: Use the **60x objective.***

*4. **Focus the image** in the microscope and then **direct the image to the camera** in order to see the image on the computer screen.*

*5. Open the **SCA® Morphology** module by clicking on "Programs" button and then select **SCA® Morphology**.*

*6. Press **Enter** in the configuration window.*

7. Check the current configuration (menu "Configuration" > "View/Edit").
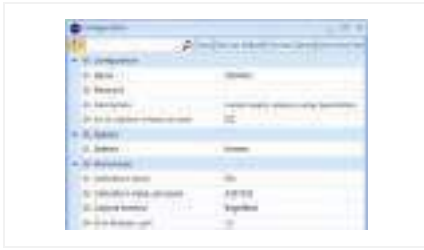
8. Observe the main analysis properties:

- *Calibration:*
  - *Standard camera: 100x*
  - *High resolution camera: 60x*
- *Capture method: Brightfield*
- *Stain: Sperm Stain, Diff-Quik, SpermBlue, etc*
- *Criteria: WHO, Kruger, Adaptative or Customized*

And press the "Save as default" button.

*9. Press the **Analyze** button in order to see a live image.*

10. Focus the image on the screen and reach the best level of contrast (adjust microscope illumination in order to achieve a homogeneous background similar to the background colour of the window).

*Then capture the fields required clicking the **Analyze** button (the software will advise you when a sufficient number of fields have been captured).*

Note For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)

Figure 1. WHO morphology analysis
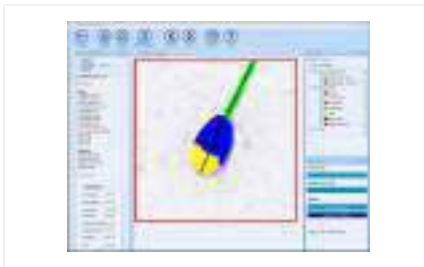
11. Double click on the sperm head to visualize the individual sperm details.
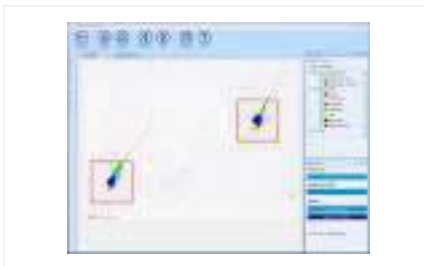
*12. After capturing the required number of fields click the **Results** button.*

13. The main window will show the general results.

General results:

1. Results of general defects

2. Sort groups classification

3. Morphology classification

4. Morphometric values

	5. Fields comparison
	6. Spermatozoa details
	7. Data summary tool
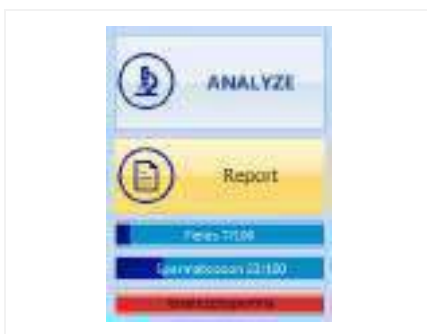
Reports:

	*1. Open the Report by clicking the **Report** button. It is also possible to create a report from the **Sample Management**: Close the analysis program, save the analysis into the database and click the Reports option from the **Sample management**.*

Report example:

2.2.3 Examples of correct and incorrect images

1. Correct image:

- *Clean background*
- *Light stain*
- *Optimum illumination control*
- *Separated spermatozoa*
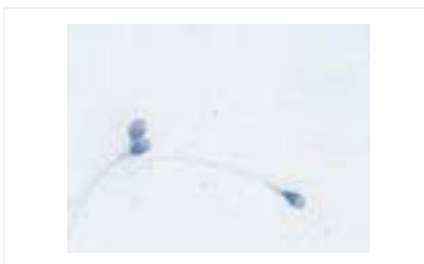
2. Incorrect image:

- *Two spermatozoa heads very close together.*
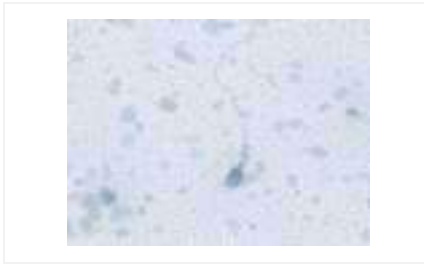
3. Incorrect image:

- *Background with a lot of detritus. Dirty background*

4. Incorrect image:

- *Too light an image (some of the midpieces cannot be analysed)*

5. Incorrect image:

- *Too dark a field (not enough contrast)*

6. Incorrect image:

- *Overstained (acrosome cannot be detected)*

7. Incorrect image:

- *One spermatozoon´s head is very close together with the tail of another.*

2.3 SCA® DNA Fragmentation

2.3.1 Protocol for DNA Fragmentation evaluation

Protocol for DNA Fragmentation analysis with GoldCyto DNA kit:
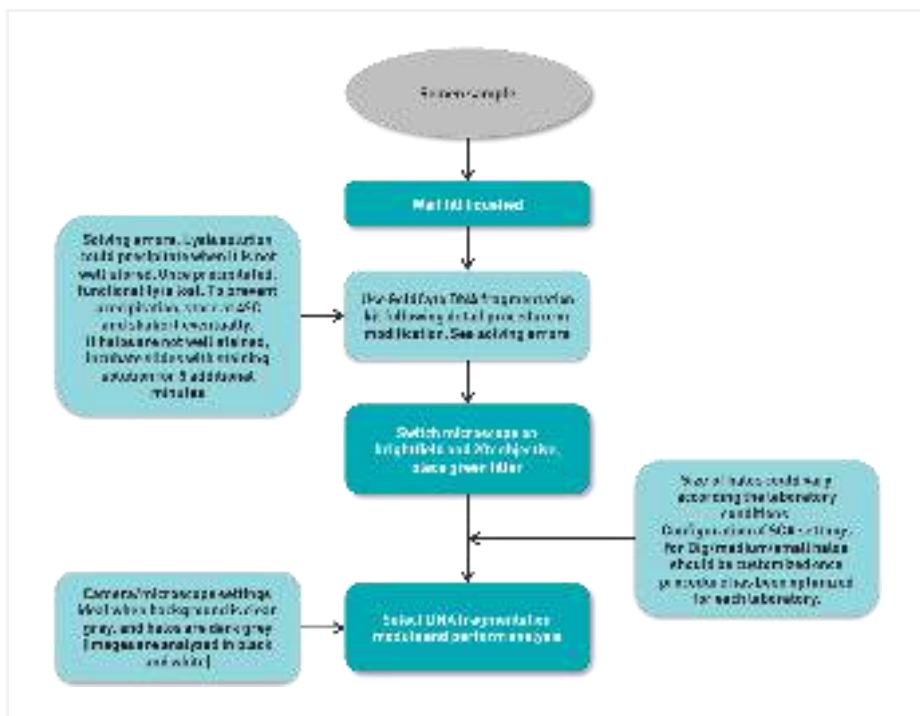
More information about sample preparation:

Follow the protocol of the **GoldCyto DNA** kit.

2.3.2 Steps to follow

1. Microscope: Insert the **green filter**.

2. Condenser: Select the **brightfield** position (A or O) depending on the microscope.

3. Place the specimen (previously prepared with the DNA Fragmentation kit Halosperm or GoldCyto DNA) onto the microscope stage.

Objective: Use the **20x objective**.

4. **Focus the image** in the microscope and then **direct the image to the camera** in order to see the image on the computer screen.

5. Open the **SCA® DNA** module by clicking on the "Programs" button and then on **SCA® DNA**.

6. Press **Enter** in the configuration window.

7. Check the current configuration (menu "Configuration" > "View/Edit").

8. Observe the main analysis properties:

- Calibration: 20x
- Capture method: Brightfield
- Disposable: Halosperm or GoldCyto DNA

And press the "Save as default" button.

*9. Press the **Analyze** button in order to see a live image on the computer screen.*

10. Focus the image on the screen and reach the best level of contrast (adjust microscope illumination in order to achieve a homogeneous background similar to the background colour of the window)

*Then capture the fields required by clicking the **Analyze** button (the software will advise when a sufficient number of fields have been captured).*

Note For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)
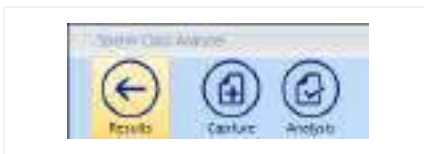
*11. After capturing the required fields click the **Results** button.*

12. The main window will show the general results.

Results window:

1. Average results of the fields analysed

2. Sort groups classification

3. Fields comparison

4. Spermatozoa details
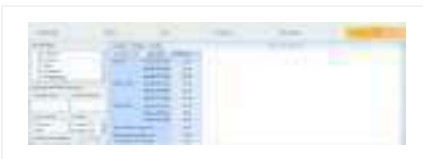
5. Data summary tool

Report:

1. *Open the report by clicking the* ***Report*** *button. It is also possible to create a report from the* ***Sample Management****: Close the analysis program, save the analysis into the database and click the Reports option from the* ***Sample management****.*

Report example:

2.3.3 Examples of incorrect images
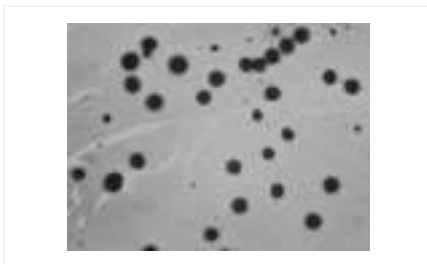
Incorrect image:

- *Not enough space between the sperms.*
- *Background too dark and dirty.*

Note: *Check the analysis properties. The selection of the limits permits different analysis, for total halo area (selected) or for halo + core area (not selected). The choice depends on the staining (if the sperms are stained too dark, you don't see the core and it's better to capture for total halo area).*

2.4 SCA® Vitality

2.4.1 Protocol for vitality evaluation

Protocol for vitality analysis with <u>FluoVit</u> kit:
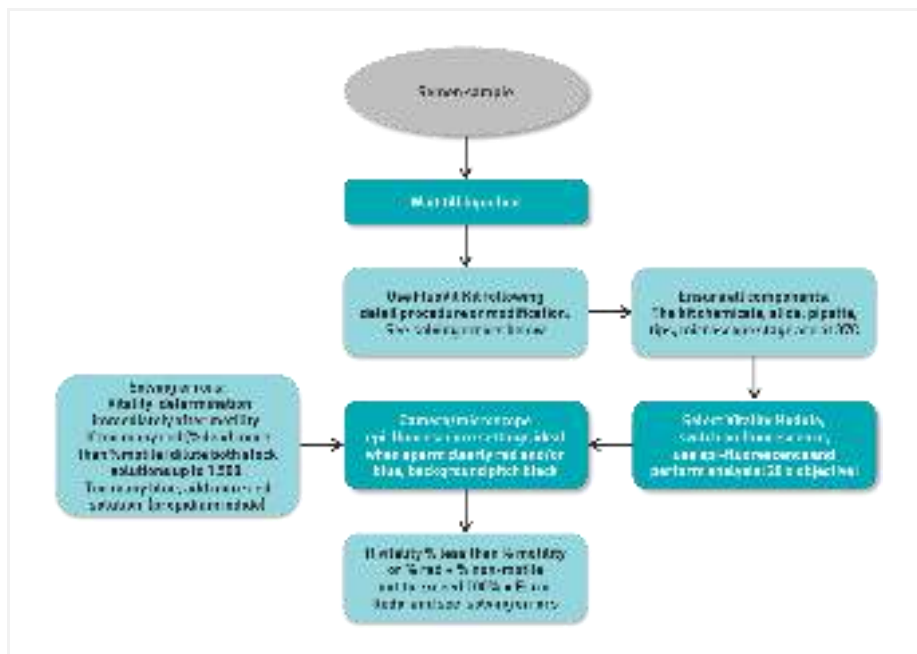
More information about sample preparation:

Follow the protocol of the **FluoVit** kit (fluorescence).

Protocol for vitality analysis with BrightVit kit:
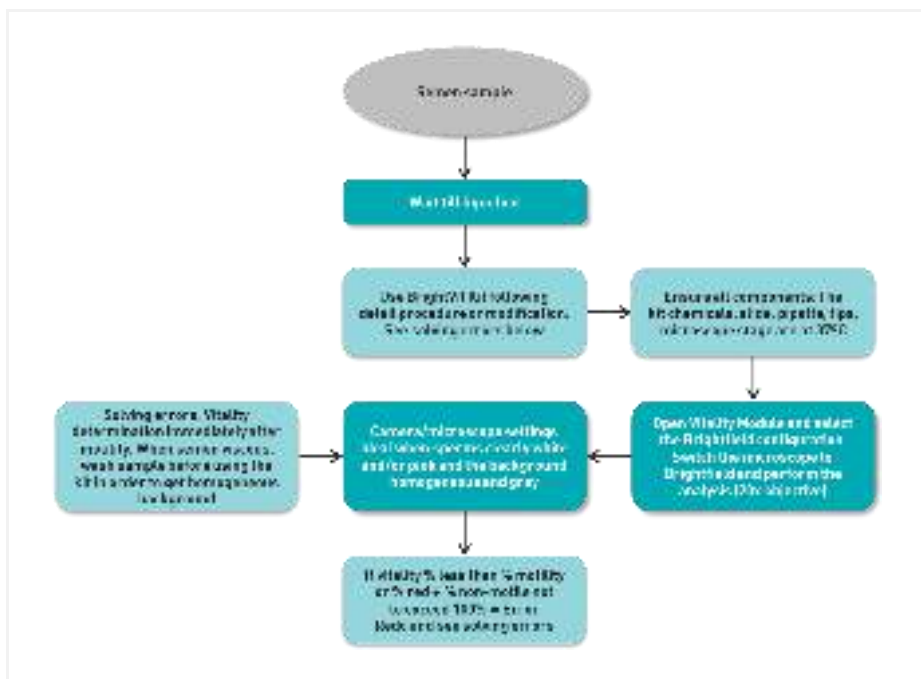
More information about sample preparation:

Follow the protocol of the **BrightVit** kit (brightfield).

2.4.2 Steps to follow

1. Place the specimen (previously stained with FluoVit or BrightVit kit) on the microscope stage.

FluoVit:

- *Microscope: Use fluorescence*
- *Fluorescence filter: DAPI filter (EX 330-380, EM 420, DM 440)*
- *Objective: Use the **20x objective***

BrightVit:

- *Microscope: Use brightfield*
- *Objective: Use the **20x objective***

*2. **Focus the image** in the microscope and then **direct the image to the camera** in order to see the image on the computer screen.*

*3. Open the **SCA® Vitality** module clicking on "Programs" button and then on **SCA® Vitality**.*

*4. Press **Enter** in the configuration window.*

5. Check the current configuration (menu "Configuration" > "View/Edit").

6. Observe the main analysis properties:

- *Calibration: 20x*
- *Capture method: Fluorescence (FluoVit) or brightfield (BrightVit)*
- *Disposable: FluoVit or BrightVit*

And press the "Save as default" button.

*7. Press the **Analyze** button in order to see the live image on the computer screen.*

8. Focus the image on the screen and reach the best level of contrast (adjust microscope illumination in order to achieve a homogeneous background similar to the background colour of the window).

*Then capture the fields required by clicking the **Analyze** button (the software will advise when a sufficient number of fields have been captured).*

Note For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)

*9. After capturing the required number of fields click the **Results** button.*

10. The main window will show the general results.

Results window:

1. Percentage of living and dead spermatozoa

2. Sort groups classification

3. Fields comparison

4. Spermatozoa details


5. Data summarization tool

Report:

*1. Open the report by clicking the **Report** button. It is also possible to create the report from the **Sample Management**: Close the analysis program, save the analysis into the database and click the Reports option from the **Sample management**.*

Report example:

2.5 SCA® Acrosome reaction

2.5.1 Protocol for acrosome reaction evaluation

Protocol for acrosome reaction analysis with FluoAcro kit:
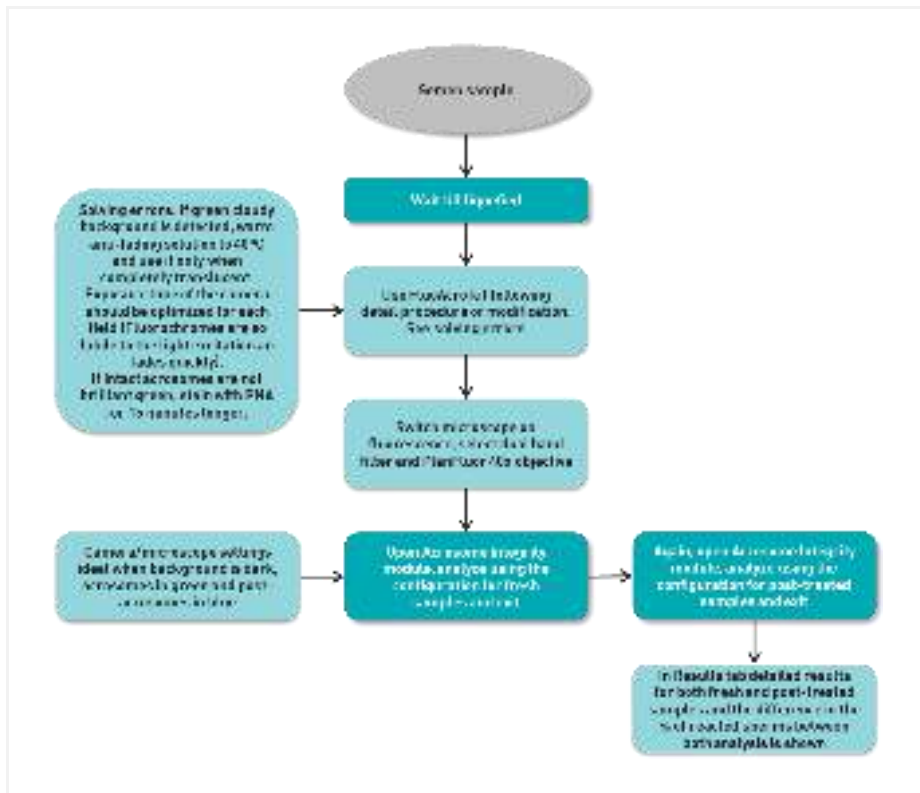
More information about sample preparation:

Follow the protocol of the **FluoAcro** kit.

2.5.2 Steps to follow

1. Place the specimen (previously prepared with FluoAcro kit) on the microscope stage.

   Microscope: Use fluorescence.

   Fluorescence filter: Dual band filter (EX 387/480, EM 433/530, DM 403/502)

   *Objective: Use the **40x plan fluor objective**.*

2. **Focus the image** in the microscope and then **direct the image to the camera** in order to see the image on the computer screen.

3. Open the **SCA® Acrosome** module clicking on "Programs" button and then on **SCA® Acrosome**.

*4. Press **Enter** in the configuration window.*

5. Check the current configuration (menu "Configuration" > "View/Edit").

6. Observe the main analysis properties:

- *Calibration: 40x*
- *Capture method: Fluorescence*
- *Disposable: FluoAcro*

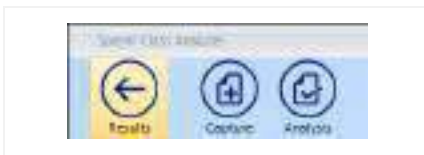*And press the "Save as default" button.*

*7. Press the **Analyze** button in order to see the live image on the computer screen.*

8. Focus the image on the screen and reach the best level of contrast (adjust microscope illumination in order to achieve a homogeneous background similar to the background colour of the window).

*Then capture the fields required by clicking the **Analyze** button (the software will advise when a sufficient number of fields have been captured).*

Note For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)

*9. After capturing the required number of fields click the **Results** button.*

10. The main window will show the general results.

Results window:

1. Percentage of intact and reacted spermatozoa

2. Sort groups classification
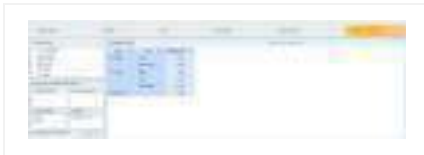
3. Fields comparison

4. Spermatozoa details

5. Data summarization tool

Report:

*1. Open the report by clicking the **Report** button. It is also possible to create the report from the **Sample Management**: Close the analysis program, save the analysis into the database and click the Reports option from the **Sample management**.*

Report example:

2.6 SCA® Leukocytes

2.6.1 Protocol for leukocytes evaluation

Protocol for Leukocytes analysis with LeucoScreen kit:
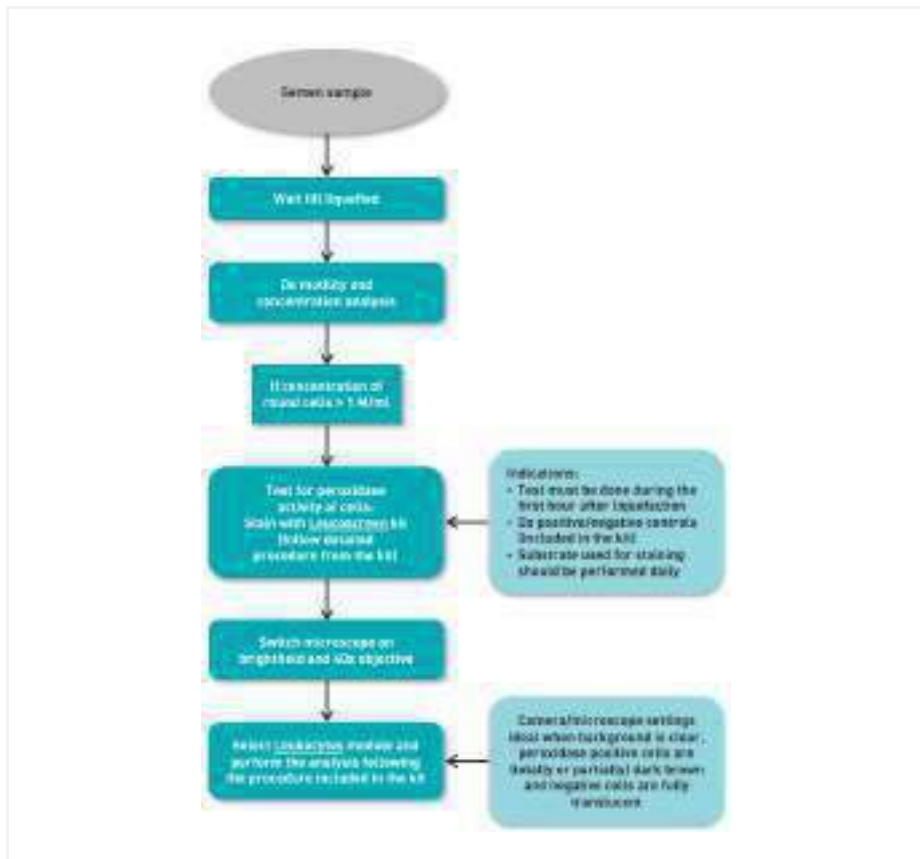
More information about sample preparation:

Follow the protocol of the **LeucoScreen** kit.

<u>The Number of fields</u>

The full screening of the sample consists in doing **4 transects** similar than this:

Do not include in the analysis those cells partially out of the transect. In no case the objective should be moved out the transect to capture a cell outside the transect limits.
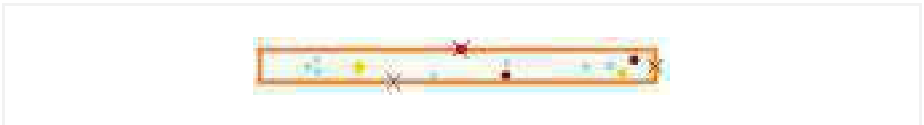
2.6.2 Steps to follow

*1. Condenser: Select the **brightfield** position (A or O) depending on the microscope.*

2. Place the specimen (previously prepared with the Leukocytes kit LeucoScreen) onto the microscope stage.

*Objective: Use the **40x objective**.*

3. **Focus the image** *in the microscope and then* **direct the image to the camera** *in order to see the image on the computer screen.*

4. *Open the* **SCA® Leukocytes** *module by clicking on the "Programs" button and then on* **SCA® Leukocytes**.

5. *Press* **Enter** *in the configuration window.*

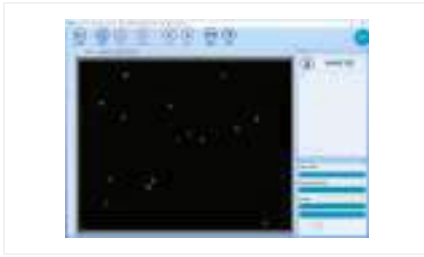*6. Check the current configuration (menu "Configuration" > "View/Edit").*
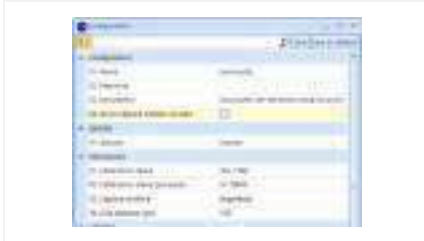
7. Observe the main analysis properties:

- *Calibration: 40x*
- *Capture method: Brightfield*
- *Disposable: LeucoScreen*
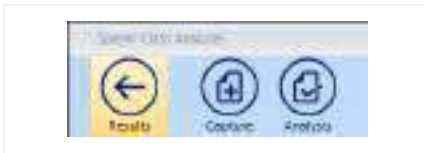
And press the "Save as default" button.

*8. Press the **Analyze** button in order to see a live image on the computer screen.*

9. *Focus the image on the screen and reach the best level of contrast (adjust microscope illumination in order to achieve a homogeneous background similar to the background colour of the window).*

*Then capture the fields required by clicking the **Analyze** button until you do 4 transects.*

Note For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)
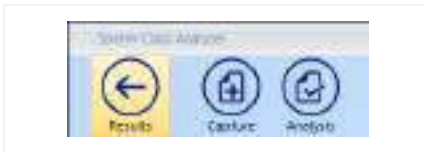
10. *After capturing the required fields click the **Results** button.*

11. *The main window will show the general results.*
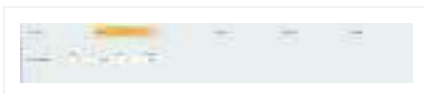
Results window:

1. *Average results of the fields analysed*

	2. Sort groups classification
	3. Fields comparison
	4. Spermatozoa details
	5. Data summary tool
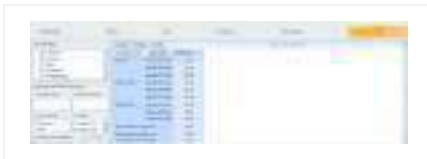
Report:

*1. Open the report by clicking the **Report** button. It is also possible to create a report from the **Sample Management**: Close the analysis program, save the analysis into the database and click the Reports option from the **Sample management**.*

Report example:

2.7 SCA® Manual Counter

SCA® Manual Counter is an application that facilitates many types of manual cell counting. It allows you to create and arrange suitable configurations based upon your own personal counters.

The counter already has some analysis set as defaults (motility, morphology, vitality, DNA fragmentation) but you can also create new counters for different analysis or different cells.

2.7.1 Steps to follow

*1. Open the **SCA® Counter** module by clicking on "**Programs**" button and then on **SCA® Counter**.*

*2. **Select the counter** you wish to use from the available counter list and press **Enter**.*

*3. Press the **Analyze** button in order to see the live image on the computer screen.*

4. Focus the image and adjust the illumination to suite.

5. Use the mouse by clicking on the chosen cell. Use the buttons on the left of the screen to choose different cells.

The total number of the selected cell will be increased automatically.
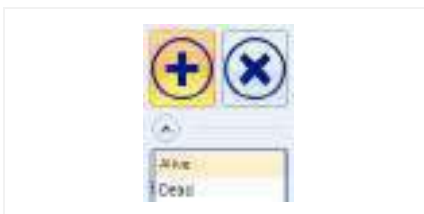
*6. You can also change the cell type with the **Add** button of the toolbox.*

*7. After counting the required number of cells click the **Results** button.*

8. The main window will show the general results.

Note For more in depth details about the Analysis functions please refer to the SCA® complete manual (SCA® installation disk)

Results:

1. General Results

2. Fields comparison

2.7.2 Manual counter configuration window

*1. In order to create a new counter for the analysis of other types of cells, click the **New** button from the **Configuration** menu.*

2. Insert a name for the new configuration.

3. Fill in the fields with the cell names that you need to count (features) and press "Save as default".

3   SCA® Results and Reports

Once you have all the analysis completed you can see the results or print several reports. From the "SCA® Sample management", select the results tab:

1. **Reports buttons**: These buttons allow you to open the final results report (summary report, complete report and customized report). The button on the right allow you to display the images from the different analysis.

2. All reports can also be opened from the **Reports** option on the top right part of the menu.

4 APPENDIX A: Laboratory guide for Motility and Concentration

4.1 Species

General Name: Human

Species name: *Homo sapiens*

Brief background: Most studies reporting on human semen are from males who have a potential fertility problem. For clinical evaluation of semen the data should be related to WHO criteria. On the basis of the WHO normal ranges males can be classified as potential fathers, sub-fertile or sterile. Addendum 1 indicates the normal ranges for semen analysis for humans.

When human semen is used for research purposes and/or high quality sperm is required, donors have to be carefully selected on the basis of criteria for semen parameters listed in Addendum 1. These parameters are also useful to decide whether there is a potential fertility problem.

4.2 Ethics and consent

Ethical approval is required for the evaluation of both patient and donor semen. A special written and signed application should accordingly go through the relevant ethics committees at the relevant organization (university, research unit, etc.) clearly stating the purpose of the investigation (e.g. clinical, research). A consent form is also required which must be signed by the patient and investigator (in our laboratory we use a consent form attached as Addendum 2 and is strongly recommended).

4.3 Methods of semen/sperm obtaining

Human semen is obtained through masturbation. The patient should be instructed to abstain for at least two days. The patient should also be instructed to wash penis,

particularly the glans penis, and dry with clean towel/paper, prior to ejaculation. A special private "masturbation room" in the clinical/research facility which is patient friendly is required (can be locked by the patient and assures 100% confidentiality, and is of utmost importance). If this is not available the donor must present analysis laboratory with a semen sample within one hour of masturbation. In each instance a sterile vial should be supplied to patient.

Wide mouthed sterile plastic containers (50 ml) with patient identification number should be used and semen produced via masturbation should be passed directly in the plastic container. The container can be kept at room temperature of 20 to 25^0C.

On arrival at the laboratory the container should be kept in an incubator/oven at 37^0C.

4.4 Semen handling and preparation

Investigators must at all times wear plastic gloves and work with plastic vials, pipettes and sharp objects such as injection needles should NOT be used (protection against pathogens such as HIV etc.). It is important to study sperm motility in semen only after full liquefaction (usually 20 minutes to one hour after masturbation) but preferably within an hour.

4.5 Choice of microscopic slides

A wide range of slides can be used for motility analysis. Normal clean high quality glass slides and a 22mm x 22mm cover slip or high quality chamber slides such as Leja can be used. These slides can be obtained as 2 chamber to 8 chamber formats (permanent cover slip attached) and the chamber volumes vary from 1 µl to 25 µl depending on the number of chambers and the chamber depth.

For human semen a four chamber Leja slide and either 10 µm to 20 µm depth is recommended. Warm Leja slide on a temperature controlled plate or stage and introduce semen (from sample in incubator) in chamber at the clearly marked opening. Semen flows in each chamber via capillary action. Very viscous samples are difficult to introduce in these chambers and a glass slide with cover slip is recommended in these cases.

4.6 Current SCA® Motility analysis

Select the SCA® Icon. Then select Motility on Main SCA® Menu. For

A. clinical evaluation of human sperm motility

Select the default setting for using the Lite SCA® version. Go to Capture and set brightness and contrast to select sperm best for positive phase contrast (sperm heads mostly white and background grey). Make sure microscope settings are optimal for critical illumination (See main SCA® manual). Preferably use a Leja slide of 10µm depth for semen and at least 20 µm for swim-up or sperm in tissue culture medium (HTF, Ham's F10).

Warm slide on warm stage or microscope stage and pipette tips in incubator at 37^0C. Use positive displacement pipette (e.g. Gilson positive replacement required for accurate sperm concentration) to insert required sperm volume from semen sample to Leja slide (1µl for 8 chambered slides to 5µl for 2 chambered slides). When using standard glass slide put 10µl semen on slide and cover with a 22mm x 22mm cover slip. This normally ensures a 20 µm depth. Under properties of motility screen make the correct selection for type of slide chosen. After inserting the correct volume into e.g. Leja slide, leave slide for 30 seconds on heating stage of microscope. Then move slide in position using x 10 objective. Quickly view different parts of slide to establish visually that motility is more or less homogenous. Capture first field immediately and quickly move slide back on to warm microscope stage (do not leave slide over opening between stage and condenser; it will lose temperature and make analysis inconsistent).

While slide "rewarms" on microscope stage, check analysed field for correct analysis. Replay and make sure settings are optimal. Then move slide in position again and capture second field and repeat as above. Analyze sufficient fields to have at least 200 motile sperm (See Fig 1 for a typical analysis of sample with about 45 x 10^6sperm/ml).

After three fields have been captured check that sperm concentration is consistent and does not vary by more than 5% to 10% among different fields. If a sample varies a lot repeat two to three further fields.

When doing sperm motility analysis in culture media ensure that there are not too many sperm on screen (sperm concentration too high). Try and work within the concentration range 3 to 20 million/ml after dilution.

SCA® measures sperm concentration accurately using Leja slides of known depth provided that the sperm concentration is approximately between 5 x 10^6/ml to 95 x 10^6/ml. If the concentration in semen is above 95 x 10^6/ml, then dilute x 20 times using positive displacement pipette in culture medium [e.g. Human Tubular Fluid (HTF) – see recipe in Addendum 3].

After basic semen analysis it is common practise to either test the quality of either swim-up sperm/or/and pelleted sperm from gradient centrifugation using Pure Sperm (See Addenda 4 and 5 for sperm washing and these two methods). See Figs 2 and 3 as examples of swim-up sperm motility patterns compared to sperm obtained through centrifugation through 40% and 80% Pure Sperm. This forms an important and integral part of testing for the best strategy of fertility management of the couple.

After sperm motility analysis of either semen or swim-up preparations there is a great deal of information on the Results screen. There are detailed results for each field analysed but also average results for all fields.

For the embryologist/clinician there is on the right hand of the results screen an indication of whether the semen sample passed the basic WHO criteria for sperm concentration and for the a and a+b motility ratings.

In addition the results screen is divided into 4 categories:

1. Show actual percentages of motile sperm in each of the a, b, c and d WHO groupings.

2. Show groupings of Rapid, Medium and Fast swimming sperm.

3. Show percentage groupings of motile sperm including hyperactivation

4. The detailed results on the motion kinetic parameters (VCL, VSL etc.).

A report can be saved in Excel format which contains all the above information in the form of tables with averages, graphic representations of the WHO criteria as well as the swimming trajectories (*.bmp format) of each field. Also all information is saved in a data base.

The total time of analysis of the semen sample for concentration and sperm motility is usually less than one minute using SCA®.

B. More advanced analyses for Research purposes

The previous analysis on the Lite SCA® version is sufficient for general clinical diagnosis and also for building a solid base for your patient records and follow ups.

However, the full Human version/Research version allows analysis in the range 50 to 100 frames per second. The Basler colour camera can analyse frame rates up to 50fps and the 602f camera can analyse frame rates up to 100fps. A frame rate of 50fps is sufficient for most research applications on human sperm in culture media/swim-up or gradient centrifugation.

The advantages of the above advanced versions are that details of individual sperm can be studied and all data recorded can be saved as a complete motility file (*.mot = can later be reanalysed using different settings under properties). A full report in Excel format with averages and details of analysis in terms of sperm motility percentages, groupings of rapid, medium and fast moving sperm as well as the kinematic parameters (e. VCL, VSL etc.) can also be saved. Finally a data file also in Excel format with the individual details of CASA parameters for each sperm can be saved. The saved data files are then available for more detailed statistical analysis when required.
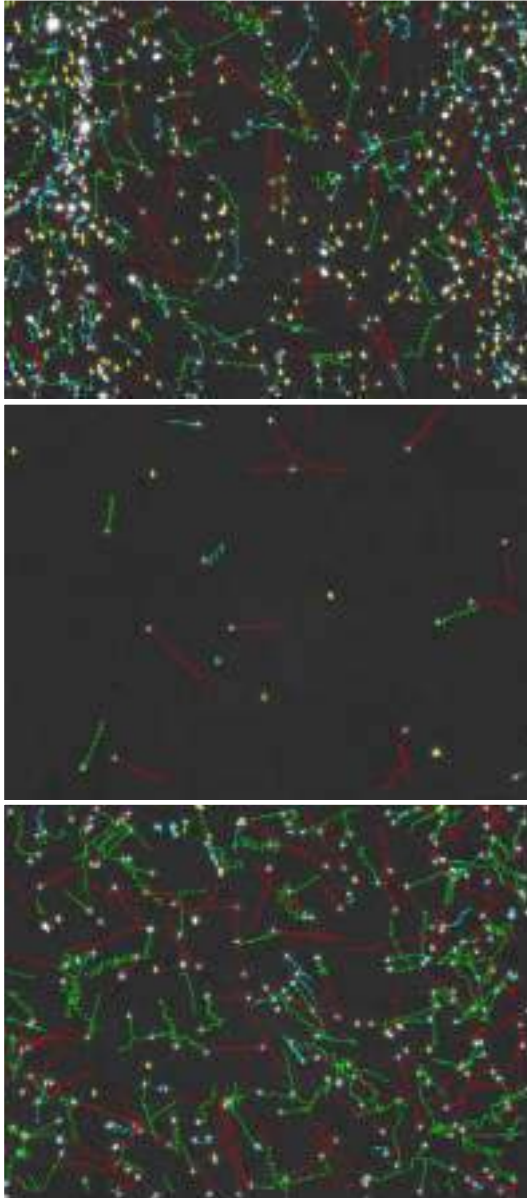
Figure 2. Top: Semen; Middle: Swim-up; Bottom: Pure Sperm. (Same sample)

4.7 Reports, Actual data, Analysis and Interpretation

It is strongly recommended that the actual motility data is saved as *.mot files. This means you can recall the original motility analysis and re-assess or re-analyse using different parameters under properties. The connectivity setting can for example be changed if fragmented tracks are present (e.g. one sperm showing two consecutive tracks with a break between the tracks) as well as the VAP setting. For very straight swimming sperm a VAP setting of 5 is acceptable. However, when sperm becomes hyperactivated and Linearity becomes smaller than 40% and 30%, VAP settings of 7 and 9 should be used. This will ensure proper smoothing of the VAP in order for more reliable detection of ALH and the other derivatives STR and WOB. VAP setting can be adjusted under Properties.

Additional advantages of first saving the actual *.mot files are numerous. They can be used for regular quality checks and checking reliability of sperm capture. Once it is ensured that the analysis is without errors, a report and data file in Excel format can be saved and printed as outlined above.

Data to be finally saved as a Data file can be further streamlined before saving. If immotile sperm needs to be omitted from a file this can be chosen from the Restrictions box under properties on Results screen (e.g. set minimum VCL to e.g. 10 and tick VCL box).

Before you present your data in table format or as graphs make sure whether it is best to do it as averages or rather as medians. For example if your data does not have a normal distribution then normal parametric statistics like averages and standard deviations should be avoided and non-parametric methods should be used. As a guideline use both the recommendations of ESHRE workshop and/or consult a statistician when unsure. The *.bmp files of the actual tracks (saved in report file or can be exported from Results screen) are useful as graphic presentations for Powerpoint presentations or research articles to show patterns of sperm motion (see Figure 22).

4.8 Relevant references

Mortimer, D, (1994).Practical Laboratory Andrology. Oxford University Press, Oxford, New York.

World Health Organization, (1992 and 1999). WHO Laboratory manual for the examination oh human semen and sperm-cervical mucus interaction. 3rd ed. and 4th ed. Reprinted 2000. Cambridge University Press, Cambridge.

4.9 Addendum 1: WHO values for semen analysis

Semen analysis: Lower minimum values/range according to WHO (1999/2000 reprinted).

Semen characteristic	Units	WHO 1999
Volume	Ml	>2
pH	pH units	7.2 – 2.8
Sperm concentration	10^6/ml	>20
Total sperm count	10^6/ejaculate	>40
Motility within 60 minutes of ejaculation	% Class a % Class a + b	>25 >50
Morphology WHO Strict Criteria	% Normal % Normal	>30 >15
Vitality	% Live	>75
Leukocytes	10^6/ml	<1.0
Immunobead test	% sperm with beads	<20
MAR test	% sperm with RBC's	<10

Semen analysis: Values for Normal, Borderline and Pathological (Mortimer, 1994)

Semen characteristic	Units	Normal	Borderline	Pathological
Volume	Ml	2.0 – 6.0	1.5 – 2.0	<1.5
Sperm conc.	10^6/ml	20 - 250	10 - 20	<10
Total sperm count	10^6 ejaculate	>80	20 - 80	<20
Motility (0.5 to 2.0 hours after ejaculation	% Motile	>50	35 - 49	<35
Progression (37ºC)	Scale 0 – 4	3 or 4	2	<2
Morphology (WHO) Head defects Midpiece defects Tail Defects	% Normal /100 sperm /100 sperm /100 sperm	>60 <35 <20 <20	35 – 59 35 – 60 21 – 35 21 - 25	<35 >60 >25 >25
Vitality	% Live	>75	50 - 74	<50

4.10 Addendum 2: Semen Donation for Research Purposes - Consent Form

I hereby give my voluntary consent to donate my sperm for research projects conducted by ……………………………………. (name of researcher) at ………………………. (name of institution).

By signing this consent form, I agree as follows:

1. My semen sample(s) will be used exclusively for research purposes. The researchers will not use my semen sample(s) for in-vivo insemination, genetic manipulation, or for in vitro fertilization of human oocytes for the creation of embryos and embryonic stem cells.
2. The researchers will routinely discard any semen they do not use for their research projects in a responsible manner as medical waste.
3. My involvement in these research projects will be kept confidential and any results will be recorded by using identification codes instead of names. Any report that the researchers publish will not include any information that will make it possible for the readers to identify me as a sperm donor.
4. I will not receive any financial benefits from future commercial development and scientific patents of discoveries made through the use of my sperm cells.

Signed this day ………… of …………….. 20….. at …………………………….

…………………………………   …………………………………………

Signature of Sperm Donor   Printed Name

(Compiled by Ms Liana Maree)

4.11 Addendum 3: Composition of Human Tubular Fluid (HTF)

Quinn *et al.* 1985 formulated HTF and Mortimer (1994) suggested the following detailed preparation protocol:

1. Dissolve the following chemicals in about 600 ml tissue culture grade water in a 1000ml volumetric flask:

   5.938 g NaCl; 0.350 g KCl; 0.049 g $MgSO_4$. $7H_2O$; 0.050 g $NaHCO_3$; 0.420 g $NaHCO_3$; 0.036 g Na pyruvate; 0.5 g Glucose; 0.003 g Phenol red; 4,766 g Hepes free acid; 3.136 ml Na lactate (60% syrup); 2.400 ml NaOH 5 N

2. Mix until dissolved
3. Separately, dissolve 0.300 g $CaCl_2.2H_2O$ in 100 ml tissue culture grade water (TCGW). Add slowly with thorough mixing to the stock HTF solution
4. Make up to 1000ml with TCGWand mix thoroughly through inversion.
5. Add 10.0g of HAS and mix carefully through inversion (be careful of frothing)
6. Adjust pH to 7.5-7.6 using 1 N HCL or 1 N NaOH (also made up in TCGW).
7. Check the osmolarity (280 – 290 MOsm/kg)
8. Filter-sterilize into strile plastic containers under positive pressure
9. Label HTF + HSA and store at 4°C for 6 months
10. Warm to 37°C before use and discard unused.

N.B. For sperm capacitation (hyperactivation studies) the Hepes and NaOH 5 N should be omitted and the $NaHCO_3$ increased to 2.100 g

4.12 Addendum 4: Sperm washing and swim-up procedures

It is well known that long term exposure of sperm to seminal plasma in semen is harmful. For either clinical studies or for experimental studies it is therefore important to separate sperm from seminal plasma. This must accordingly be done to enhance sperm physiology. Sperm can be diluted about five times in a wide range of culture media such as HTF and Ham's F10. The disadvantage of simple dilution is that both good and poor quality sperm will be present as is the case in semen. For both clinical use (IVF) and research it is important to be able to separate good quality motile sperm from the rest. For this purpose sperm washing techniques exist.

While a wide range of methods are available that involves several steps of washing the following procedure is recommended:

Simple swim-up technique:

1. Use two to three tubes to increase the yield of motile sperm. Put typically 500 µl - 1000 µl of tissue culture medium in each tube (plastic sterile Falcon tube) at 37°C.
2. Use Gilson or equivalent pipette and withdraw 200 µl to 250 µl of semen that was maintained at 37°C and using a pipette tip at 37°C.
3. Insert tip carefully through the medium containing the culture medium in the Falcon tube to bottom of tube.
4. Very slowly release semen at bottom of tube. When all semen is released as a "pellet", slowly withdraw pipette without disturbing the "sperm pellet" at the bottom. Motile sperm will now swim into the medium. Within about 10 minutes progressively motile sperm can be collected at the very top of the tube. However, the motile sperm yield is better after 30 minutes and one hour. Traditionally sperm harvesting from the top motile fraction is sampled after an hour. In good samples the sperm population is very homogenous in terms of their sperm kinetic parameters and the VCL is usually higher than 100 µm/second at 50fps.

4.13 Addendum 5: Differential centrifugation and sperm pellet with motile sperm

Historically differential centrifugation using Percoll, has been used to separate different motile fractions of sperm. The major advantage of this method is that it fully separates motile sperm from seminal plasma, immotile sperm, leukocytes and bacteria. The major disadvantage of this method is that there are minute Percoll-silica coated particles and endotoxins which pose problems in terms of insemination procedures.

Several new media have been developed that is now commonly used. The one that is recommended is Pure Sperm. It can be bought as 40% and 80% or as 100% stock. The 100% stock can then be diluted to respectively 80 and 40%. The advantage of the 40% and 80% solutions are that they are made up in sterile balanced salt solution.

Method:

1. Take sterile plastic conical Falcon centrifuge tube 2095 and pipette 1.5 ml of 80% Pure sperm.
2. Carefully and slowly pipette/layer 1.5 ml of 40% Pure Sperm on top of the 80% Pure Sperm.
3. Layer 1.5 ml of semen on top of 40% Pure Sperm.
4. Centrifuge the gradients at 300g for 20 minutes. A soft pellet forms at bottom of tube.
5. By using a Pasteur pipette remove the upper layer of seminal plasma and upper interface material and discard.
6. Use a sterile Pasteur pipette to remove soft pellet and insert in a new Falcon tube containing about 5 ml of HTF (see recipe, Addendum 3) or preferred culture medium and mix carefully. Withdraw an aliquot and measure sperm concentration with SCA®.
7. In the mean time centrifuge the 5ml of HTF with sperm at 600g for 10 minutes in Falcon centrifuge tube.
8. Remove the supernatant and leave about 200 to 250 µl of sperm/HTF medium. The required volume can now be withdrawn to dilute/use required sperm concentration.

N.B. Scale volumes of Pure Sperm and semen down if required or in case of low sperm volume or for experimental studies.

5 APPENDIX B: Laboratory Guide for Morphology

The sample preparation is the most important step in the morphology analysis.

If the raw sample is **greater than 100 M/ml, dilute** according to standard protocol.

If the raw sample is **less than 1 M/ml**, you can **concentrate** according to the following procedure:

1. Determine the concentration of the sample (**C**).

2. Transfer 500 µl of sample to a centrifuge tube and add 1000 - 2000 µl of washing medium (e.g.Hams F10 + 1% BSA).

3. Mix gently by swirling or agitate by running tube once along the top of an empty test tube rack (no vortex).

4. Centrifuge sample for 5 minutes at 200 g.

5. Carefully aspirate the supernatant - being extremely careful. The volume of the remaining pellet should be approximately 10 µl.

6. Resuspend the pellet using the volume determined by the following equation:

   $V = 5C$ µl

   Thus, if a sample had an initial concentration (C) of 20 M/ml, then:

   $V = (5)(20) = 100$ µl

   So 100 µl of washing medium would be used to resuspend the pellet.

Samples that are concentrated using the above procedure may not have to be washed. However, if the smear shows excessive debris, additional washing will be necessary.

Washing the sample

If the sample shows a lot of detritus is convenient to wash it:

1. Place 500 µl of sample into a microcentrifuge or conical tube.

2. Add 500 µl of washing medium (*Hams F10 + 1% BSA*).
   If *Percoll* is used, the specimen must be washed completely Percoll-free before staining, otherwise staining will be incomplete.

3. Swirl or Repeat pipette gently to mix.

4. Centrifuge for 5 minutes at 200 g.

5. Remove supernatant by aspiration.

6. Resuspend pellet in 500 µl of washing medium.

7. Repeat pipette gently to mix.

8. Centrifuge a second time for 3 minutes at 500 g.

9. Remove supernatant by aspiration.

10. Resuspend pellet with 125 µl of washing medium.

11. Repeat pipette gently to mix. The sample is now ready to use to make a smear.

If sample is extremely debris laden, repeat steps 8-11 again

Prepare at least two smears from each sample as follows:

1. Wash pre-cleaned microscope slides with alcohol. Rinse with distilled water and wipe dry. Warm slides on the slide warmer set to 37°C.

2. Place a 5 µl droplet of sperm near the frosted end of the microscope slide.

3. Using another slide held obliquely to the first (approximately 30° angle), touch it against the drop of sample (away from the frosted end).

4. After the droplet has spread along the junction of the slide, gently pull the second slide away from the drop along the length of the first slide.

   Note: If specimen has a very low initial concentration (10 M/ml or less), the droplet should be spread in a streak down the center of the slide to conserve sufficient sperm density.
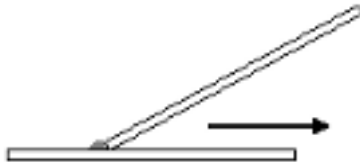
Figure 3. Correct smear scheme

Placed prepared smear on the slide warmer and allow to air dry (approximately 15 minutes).

Staining with Dif-Quik (recommendable)

Reagents & Supplies Needed

- *Diff-Quik Kit*: consisting of Fixative, Stain Solution #1 and Stain Solution #2

- Distilled Water

- Coplin Staining Jars

- Paper towels

Diff-Quik Staining Protocol

1. Fix smear by immersing slide in *Diff-Quik* Fixative **1 second 5 times**
2. Stain sperm by immersing fixed slide in *Diff-Quik* Solution #1. **1 second 5 times**
3. Stain sperm by immersing slide in *Diff-Quik* Solution #2. **1 second 5 times**
4. Rinse slide in distilled water. **1 second 5 times**
5. Allow to dry

If re-examination of slides is required, coverslipping will help prevent deterioration of the smear due to repeated exposure to immersion oil. It will also help to maintain a clean objective. Though coverslipping is optional, it is recommended.

Mounting a coverslip onto the slide requires the use of a mounting medium such as the *Eukitt* mounting medium.

Coverslipping Procedure

1. Place 3-4 drops of mounting medium along the center of the dried slide.
2. Carefully place a clean coverslip (50mm x 22mm) on the slide.
3. Press the coverslip gently to distribute the mounting medium.
4. Allow to dry sufficiently

Advises for morphology analysis:

The following images show different kind of captures:

1. Correct image

Figure 4. Correct image

- Clean background
- Light stain
- Optimum illumination control
- Separared spermatozoa

2. Incorrect image

Figure 5. Incorrect image (agloutination)

- Two spermatozoa heads very close together.
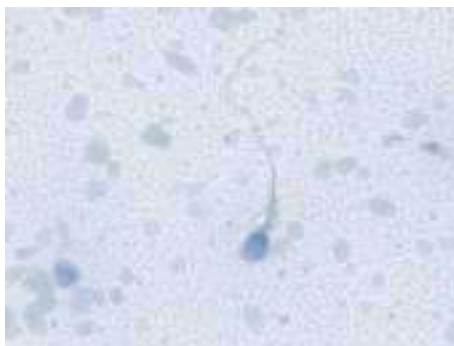
3. <u>Incorrect image</u>

Figure 6. Incorrect image (dirty)

- Background with a lof of detritus. Dirty background

4. <u>Incorrect image</u>

Figure 7. Incorrect image (high brightness)

- Too light image (some of the midpices cannot be analysed)

5. <u>Incorrect image</u>

Figure 8. Incorrect image (dark)

- Too dark field (not enough contrast)
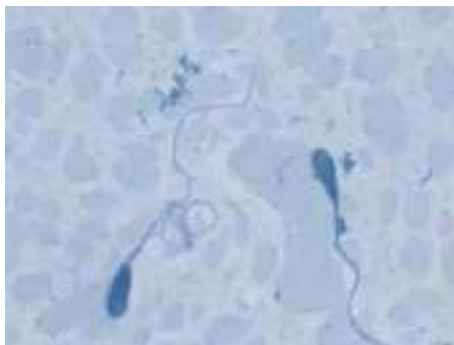
6. <u>Incorrect image</u>

Figure 9. Incorrect image (too stained)
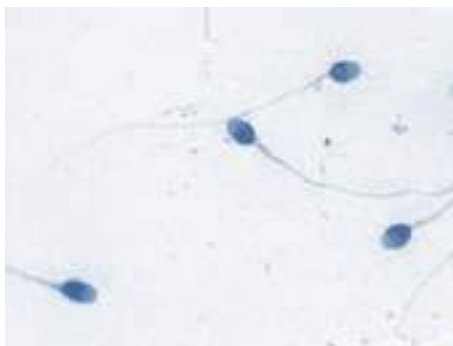
- Too stained (acrosome cannot be detected)

7. <u>Incorrect image</u>

Figure 10. Incorrect image (spermatozoa too close)

- One spermatozoon´s head is very close together with the tail of another.

<u>W.H.O. SPERM MORPHOLOGY TYPES</u>

Normal

Figure 11. Normal spermatozoon

Defects

1) Head Defects

     a)   Brush (paintbrush)
     b)   Pyriform (Narrow base)
     c)   Round
     d)   Amorphous
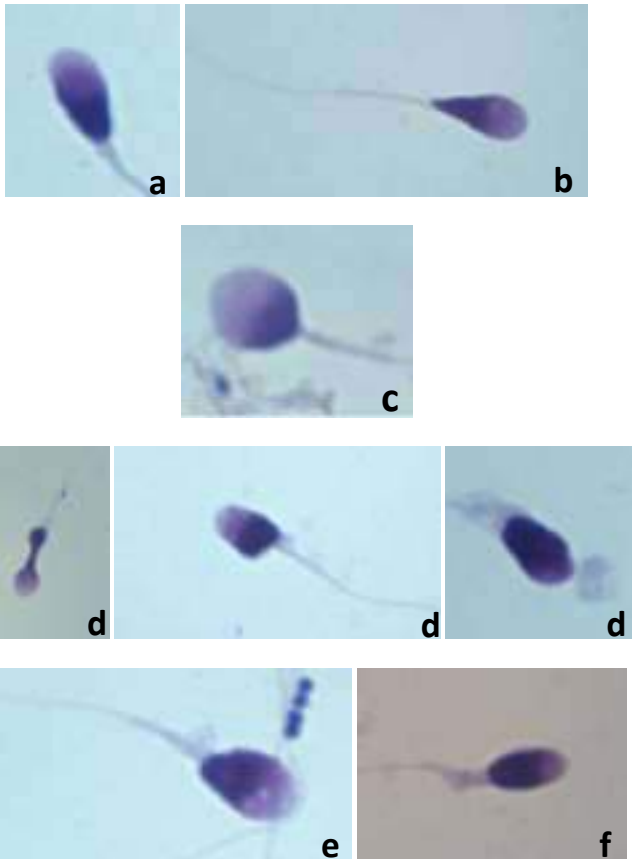   e)   Vacuolated
     f)   Small acrosome area

Figure 12. Spermatozoa with defects

2) Neck and Midpiece defects

a) Thick midpiece

b) Thin midpiece
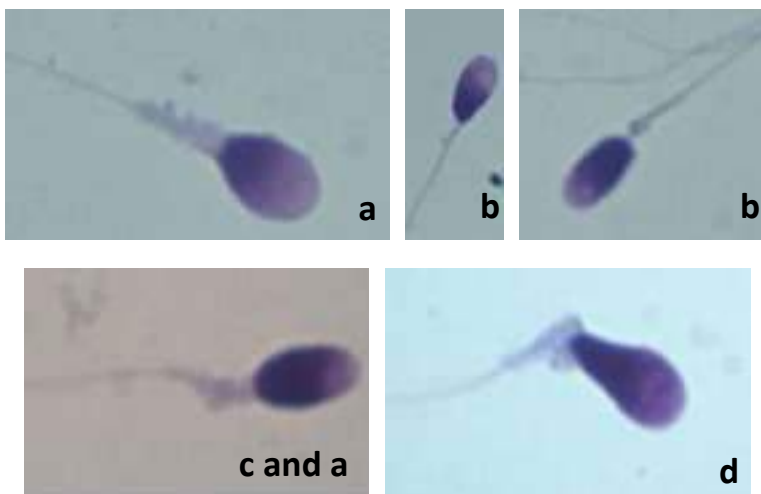
c) Asymmetrical insertion of the midpiece

d) Bent neck

Figure 13. Spermatozoa with tail and intermediate piece defects

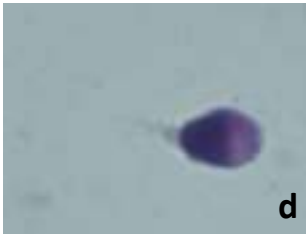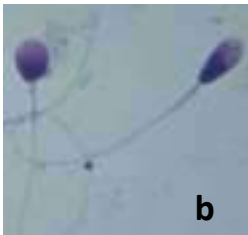## 3) Tail defects
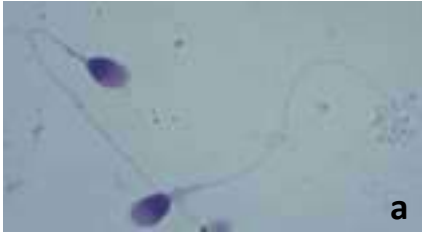
a) Bent

b) Coilled

c) Short

d) Without

Figure 14. Spermatozoa with tail defects
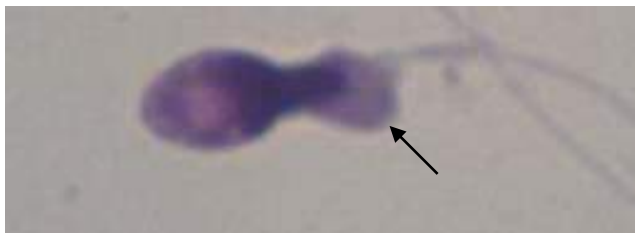
4) Cytoplasmic droplets

Figure 15. Spermatozoon with cytoplasmic droplet

<u>**Examples:**</u>

Example 1: More than 1 defect

Figure 16. Examples: a) Paintbrush head, small acrosome and high imtermediate piece b) Pyriform head, cytoplasmic droplet and coiled tail

Example 2: These are less common types: elongated head (a) or small head without acrosome (b).

Figure 17. Examples: a) Elongated head b) small head without acrosome
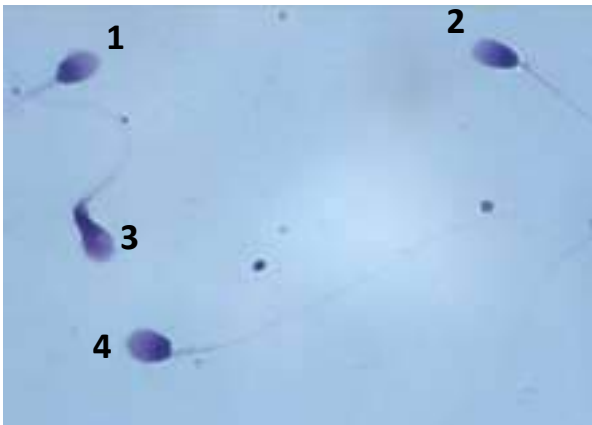
Example 3: Several defects

Figure 18. Examples: 1) Paintbrush head 2) slightly paintbrush head 3) pyriform head and bent neck 4) normal

1) Head defect: slightly brush

2) Head defect: slightly brush

3) Defects: a) head: narrow base (pyriform), b)neck: bent

4) Normal
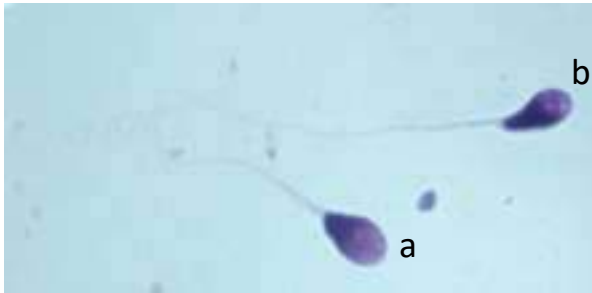
Example 4: Several defects

Figure 19. Examples a) pyriform head slightly vacuolated b) asymmetrical insertion midpiece and abnormal tail

All Images: Diff Quik Staning. Capured with SCA® - Sperm Class Analyzer® - CASA

Aspects to consider in order to obtain the results correctly

It is important to remember that the semen is a cellular non-homogenous solution; therefore, it is absolutely necessary to homogenize it, by gently moving, just before picking-up the aliquot of sample. It is not sufficient to homogenize it only once at the beginning of the analysis.

- Conditions suitable for performing the smear: In order to optimize it, we will place the slide that we use for spreading, such that it forms a 45º angle with the slide that contains the sample drop. The drop has to be spread over the slide, by making a soft and continuous movement. Since the semen sample could be considerably viscous, the smearing may not be possible, therefore you will have to take special precautions when performing this operation, otherwise, the cells may aggregate all in the same place. It's important to dry the slides before starting the staining (wait between 10 and 20 minutes).

- Staining: Usually, it is recommended to follow the instructions on the staining kit used, although you need to keep in mind that the products could become old, which (except for haematoxylins) results in a poor quality of staining. Therefore, it is convenient to have standard images made from samples stained using a fresh kit, in order to modify the staining times, when necessary to obtain equivalent results.

- Microptic recommends SpermBlue stain and Diff-Quik. Other staining solutions can be used but those two are the ones giving the best results for capturing.

- Note: It is required to capture and analyze all the sperms that do not show alterations due to smearing, or appear superposed with other cells or detritus from the background. A special case is the seminal plasma which, if it is highly proteic, may hide the sperm tails. In this case, search for the cells that are in good capture conditions, at the edges of the preparation.

- Changes in the classification of the spermatic morphology: The automatic classification given by the system follows the WHO criteria (2010), Kruger Tygerber criteria or David classification. It is necessary to take into account that the sensitivity given by the SCA® is of the order of a thousand of microns. Therefore, changes as subtile as that, could consider a cell either normal or abnormal. Therefore, in case a cell is classified in disagreement with the operator, it will be sufficient to change its classification on the screen, in order for that modification to appear in the reports.

- There is also an option for user defined criteria, the user can change and save the morphology and morphometry matrix, and therefore classify the spermatozoa according to it.