Standard Operating Procedures (SOP) for: **CYTOLOGY – Morphology Analysis**			
SOP Number:	**1.0**	Version Number:	**1**
Effective Date:		Review Date:	

Author:	**Lorraine Frew**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown – HABSelect Co-Lead Laboratory Investigator**

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: - To clearly document the process of performing morphology analysis on modified papanicolaou stained sperm. Objective: - To ensure delegated research staff members conduct this analysis according to standardized protocol.

SOP Text

Sperm morphology evaluation is conducted according to Tygerberg strict criteria. In these criteria a typical spermatozoon is defined as having an oval form with a smooth contour and a clearly visible and well-defined acrosome with homogeneous light-blue staining. The tail should be apically inserted without any abnormalities of the neck/midpiece and tail region and no cytoplasmic residues at the neck/midpiece or tail regions.

The typical sized acrosome should cover 30-60% of the anterior part of the sperm head. The typical sized head will measure between 3.0 and 5.0 µm in length and 2.0-3.0 µm in width. The midpiece should not be longer than the 1.5 x the length of a typical head and about 1 µm thick. The tail should be about 40-50 µm long and without any sharp bends. No cytoplasmic material should be present at the head/neck junction or along any part of the tail, however a smooth droplet/residue of <30% of the normal sized sperm head is still regarded as normal.

Atypical spermatozoa are those that deviate from the defined criteria for morphologically ideal spermatozoa, due to differences in size, structure, or form. The assessment relates to the main regions of the spermatozoon and abnormalities will be tallied for each of the four categories;

- Head defects
- Neck/Midpiece defects
- Tail defects
- Cytoplasmic droplet defects

An abnormal sperm may have abnormalities in one to four of these categories.

Equipment and materials required

- Microscope with bright field illumination
- Cell counter
- Immersion oil
- Lens cleaning paper

Protocol

1. Scan the smear to observe the spreading of the spermatozoa in the smear, the staining quality and the presence of round cells.

2. Identify suitable areas for the performance of the sperm morphology evaluation.

3. Sperm morphology should ideally be performed in more than one area to increase the accuracy and evaluation.

4. Assess at least 200 spermatozoa.

5. Record the morphological appearance of each spermatozoon as normal or abnormal. Assign four buttons on the counter for each of the four types of abnormalities. When a spermatozoon is scored as abnormal the button for the specific abnormality is depressed; if more than one defect is present the buttons for each defect are depressed simultaneously. In this way all the defects of the spermatozoon will be scored, but eh abnormal spermatozoon will only be counted once.

Calculation of results

The proportion of typical spermatozoa is calculated by dividing the number of spermatozoa assessed as "typical" by the total number of spermatozoa assessed. The result is given as an integer percentage (no decimal places e.g 5% not 4.8%).

Proportion of abnormal sperm and sperm with defects in the four categories are presented in the same way. While the total number of typical and abnormal cells will add up to the total number assessed, the sum of all abnormalities will be greater than the total number of spermatozoa that have been assessed.

Each morphologically abnormal spermatozoon will have any one of the four types of abnormalities described, or any combination of two to all four abnormalities. To reflect this The Teratozoospermia Index (TZI) is used as an indication of the average number of defects per abnormal spermatozoon. To calculate the TZI, the sum of all recorded abnormalities is divided by the total number of abnormal spermatozoa. Since any given spermatozoon can have between one and four abnormalities, the TZI result will always be between 1.00 and 4.00.

Example Presentation of results;

200 sperm assessed in total:
10 normal sperm = 5%
190 abnormal sperm

- 186 with head defects = 93%
- 33 with neck/mid-piece defects = 16%
- 48 with tail defects = 24%
- 8 with abnormal cytoplasmic material = 4%

TZI = (186 + 33+ 48+8)/190 = 275/190 = 1.45

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Standard Operating Procedures (SOP) for: **Cytology – Modified Papanicolaou Staining**			
SOP Number:	**02**	Version Number:	**1**
Effective Date:		Review Date:	

Author:	**Lorraine Frew**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown – HABSelect Co-Lead Laboratory Investigator**

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: - To clearly document the process of performing Modified Papanicolaou Staining Protocol using an automated slide staining machine. Objective: - To ensure delegated research staff members conduct this assay according to standardized protocol.

SOP Text

Equipment Required

- Leica Autostainer XL
  Leica Biosystems
  Equipment currently used in Birmingham – Serial # XL074996

Solutions Required

- Ethanol Absolute
  Used to make 50%, 70%, 80% and 95% EtOH
  VWR Chemicals, 20821.330

- Harris Haematoxylin
  Surgipath, Leica Biosystems, 3801560E

- Scott Tap Water Substitute
  Surgipath, Leica Biosystems, 3802901E

- 0.5% HCl – 1ml concentrated HCl in 200ml H_2O

- Papanicalou Stain Orange G6
  Surgipath, Leica Biosystems, 3801660E

- EA-50
  Surgipath, Leica Biosystems, 3801620E

- Clearium Mounting Medium
  Surgipath, Leica Biosystems, 3801100

Additional Items

- Coverslips – 2.4 x 50 mm

Protocol

1. Fix slides for at least 10-15 minutes in absolute EtOH
2. Transfer slides to the staining rack
3. Load slides in to AutostainerXL
4. Slides will follow pre-programmed protocol as follows;

Step	Reagent	Exposure
1	80% EtOH	10 dips = 10 seconds
2	70% EtOH	10 dips = 10 seconds
3	50% EtOH	10 dips = 10 seconds
4	Distilled water	10 dips = 10 seconds
5	Harris' Hematoxylin	5 minutes
6	Rinse in running water	3 minutes
7	0.5% HCl	2 dips = 2 seconds
8	Rinse in running water	5 minutes
9	Scott's Tap water	1 minute
10	Rinse in running water	2 minutes 30 seconds
11	50% EtOH	10 dips = 10 seconds
12	70% EtOH	10 dips = 10 seconds
13	80% EtOH	10 dip s= 10 seconds
14	95% EtOH	10 dips = 10 seconds
15	Orange G6	5 minutes
16	95% EtOH	5 dips = 5 seconds
17	95% EtOH	5 dips = 5 seconds
18	EA-50	5 minutes
19	95% EtOH	5 dips = 5 seconds
20	95% EtOH	5 dips = 5 seconds
21	95 % EtOH	5 dips = 5 seconds

5. Once the protocol is complete unload slides from the Autostainer XL
6. Mount slides with Clearium Mounting Solution and allow to dry

	Responsibility	Activity
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Standard Operating Procedures (SOP) for: **TUNEL Assay**			
SOP Number:	**03**	Version Number:	**1**
Effective Date:		Review Date:	

Author:	**Lorraine Frew**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown – HABSelect Co-Lead Laboratory Investigator**

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: - To clearly document the process of performing TUNEL assay Objective: - To ensure delegated research staff members conduct this assay according to standardized protocol.

SOP Text

Kit Required

- *In Situ* Cell Detection Kit, Fluorescein
  Roche, 11 684 795 910
- DNase I recombinant

Solutions Required

- Phosphate Buffered Saline (PBS)
  (PBS Tablets, Sigma, P4417, dissolved in dH_2O)
- 2mM DTT (Dithiothreitol) in PBS – **Freshly Prepared**
  (DL-Dithiothreitol, Sigma, D9163-5G, stored at 5ºC)
- Permeabilaztion Solution; 10 mg Sodium Citrate, 10µl Triton x-100 in 10 mls dH_2O – **Freshly Prepared** (Sodium Citrate, Sigma, S4641. Triton X-100, Sigma, X100-100.)
- PFA (Paraformaldehyde) – 4% PFA in PBS, pH 7.4
  (Paraformaldehyse, Powder, 95%, Sigma,158127)
- DNAse Diluent – 50mM Tris-HCl, pH 7.5, 1mg/ml BSA)
  (Tris (hydroxymethyl)aminomethane,Sigma, 252859 . Bovine Serum Albumin, Sigma, A7906).

- Clearium Mounting Medium
  Surgipath, Lecia Biosystems, 3801100

Additional Items

- Coverslips – 2.4 x 50 mm
- Humidified Chamber
- Ice

Protocol

1. Incubate slides in 2mM DTT (in PBS) for 45 minutes at Room Temperature:
   Place slides in a coplin jar containing 2mM DTT

2. Wash slides with PBS – 1 x 5 minute wash:
   Place slides in coplin jar containing PBS

3. Fix slides with PFA for 15 minutes on ice – *This must be carried out within a fume hood* ;
   Places slides in coplin jar, placed on ice, containing PFA (as described in solutions)

4. Wash slides with PBS – 3 x 5 minute wash;
   Place slide in coplin jar containing PBS, remove in to fresh PBS at 5 minute intervals x 3

5. Incubate slides in Permeabilization Solution for 2 minutes on ice;
   Places slides in coplin jar, placed on ice, containing Permeabilization solution (as described in solutions)

6. Wash slides with PBS – 2 x 5 minute wash;
   Place slide in coplin jar containing PBS, remove in to fresh PBS at 5 minute intervals x 2

7. Positive control samples need to be included in each experimental set up. Make DNase I recombinant solution to a concentration of 3U/ml in DNase Diluent (see solutions above), dilution will dependent on stock concentration of DNase I recombinant.

8. Add 25µl DNase I solution to 2 slides and add a coverslip to each slide. Place the slides in a humidified chamber and incubate for 60 minutes at 37ºC. All other slides remain in PBS during this incubation period.

9. Wash slides with PBS – 2 x 5 minute wash;
   Place slide in coplin jar containing PBS, remove in to fresh PBS at 5 minute intervals x 2

10. From the *In Situ* Cell Detection Kit remove one pair of tubes (vial 1: Enzyme Solution, and vial 2: Label Solution) – This is sufficient for staining 22 samples by using 25µl TUNEL reaction mixture per sample and 2 negative controls by using 25µl Label Solution per control.

11. Remove 50µl Label Solution (vial 2) for 2 negative controls. Add total volume (50µl) of Enzyme Solution (vial 1) to the remaining 450µl Label Solution in vial 2 to obtain 500µl TUNEL reaction mixture. Mix well to equilibrate components.

12. Add 25µl of TUNEL mixture to each slide

13. Add a coverslip to each individual slide and place the slides in a humidified chamber. Incubate the slides for 60 minutes at 37ºC in the **DARK**

14. Wash slides with PBS – 3 x 5 minute wash;
    Place slide in coplin jar containing PBS, remove in to fresh PBS at 5 minute intervals x 3

15. Mount slides with STILL TO BE DECIDED and allow to dry (**protect from direct light**) – *This must be carried out within a fume hood*

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Standard Operating Procedures (SOP) for: General sperm sample processing for downstream analyses.			
SOP Number:	**04**	Version Number:	**1**
Effective Date:		Review Date:	

Author:	**D. Miller**
Reviewed by (Name/ Position):	**J.Kirkman-Brown**

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: To prepare sperm cells for slide-based downstream assays. **Objective:** To provide continuity in sample processing to ensure that the numbers of sperm being loaded on to assay slides is both useful to the assay (in terms of sperm numbers) and the operator (in terms of workload and demand) and gives consistent results. **Scope:** To allow the rapid assessment of at least 100 sperm nuclei in each sample.

SOP Text

Introduction to technique

In order to support the downstream analysis of sperm samples acquired by HABSelect (for example), it is necessary to process the samples such that cell numbers are adequate for the purpose of the tests. This SoP deals directly with processing to support slide-based assays including Acridine Orange (SoP No), Aniline Blue (SoP No) staining and Tunel labelling. It can in principle be applied to many other slide based assays including general staining and immunocytochemistry. Comet (SoP No) and Halo (SoP No) assays have their own sperm prep protocols to follow.

Reagents and equipment required

Unless otherwise stated, all working stocks and solutions MUST be made up with Analar grade reagents.

1. A supply of 15 ml Falcon tubes for centrifuging samples.
2. A set of 1000 µl and 200 µl range pipettes with disposable tips.
3. A supply of phosphate buffered saline (PBS), pH 7.4 (freshly prepared from tablets or powder (Sigma). Once made up, this can be stored for up to 10 days at 4°C.
4. Microscope slides, acid washed and coated with poly-L-lysine (Supplier?)
5. Copling vessels (for fixing samples and washing slides).
6. SpermBlue staining agent (Microptic) or equivalent if image capture is required.
7. Microscope equipped with x25 and x40 objectives. It is preferable to use the same microscope that is to be used for all downstream analyses.

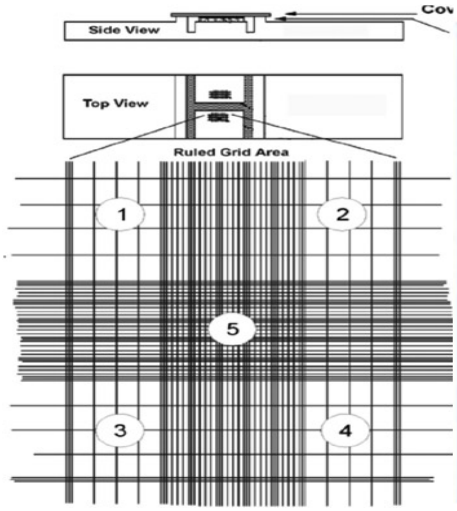
Sample Processing

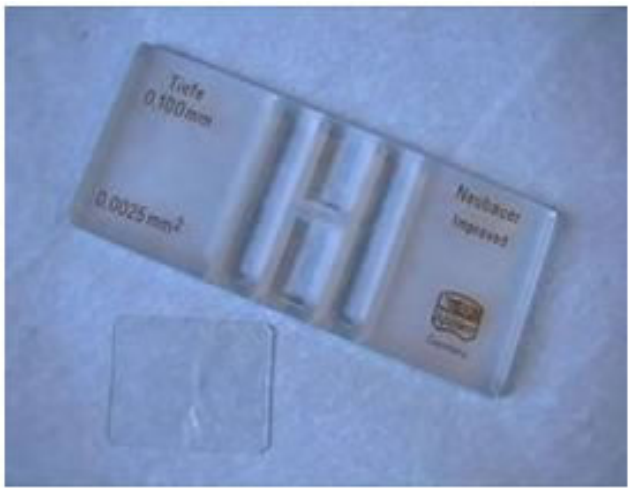
All samples arrive frozen in 500 µl aliquots. They should be stored at -80°C prior to processing.

1. Rapidly thaw sample out by placing in a 37°C water bath. Mix gently and transfer to a

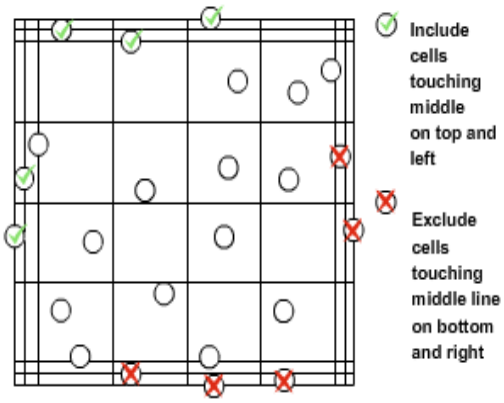
15 ml Falcon tube. Slowly add 500 µl of PBS (room temp), continuing to mix gently. Pellet sperm by centrifugation at 500g for 10 min at room temperature and remove supernatant.

2. Using a 100 µl Gilson-type pipette, gently resuspend the pellet in 100 µl of PBS held at room temperature and add another 400 µl of PBS, mixing gently.
3. Centrifuge as before and resuspend pellet in 150 µl of HBS.
4. Use a Neubauer chamber to count sperm (see below). The chamber comes with a thickened glass coverslip that is put in place *before* adding the sample. Using the edges of your thumbs, carefully slide the coverslip over the surface of the chamber, applying firm but not excessive pressure over the whole surface of the coverslip. A coverslip that is correctly positioned will generate Newton's rings (iridescent colours) around its edges when the chamber is held at a slight angle and you look across the coverslip.
5. Add 10 µl of your sample to the edge of the coverslip and allow the liquid to be drawn between the coverslip and chamber by capillary action.





6. Place the chamber beneath a x25 objective and bring the sperm into focus on the first (1) of the corner squares (1, 2 , 3 or 4). Switch to x40 and using a manual counter, count the cells in the square, moving from left to right, row by row and omitting cells contacting the right and lower edges (red crosses). The example below shows an optimal cell density that facilitates easy counting. Repeat for each of the three other corner squares.



7. As each of these big squares represents a volume of 0.0001 ml (0.1 x 0.1 x 0.01), the total (*C*) of cells per ml is given by the formula $C = \frac{N}{4} x 10^4$. So, if *N* is 100, then the sperm concentration is 25 x 10^4 / ml. If the sample measured was diluted from the original sample, the dilution factor must be taken into account. For example, if the sample was diluted 1:10 prior to counting, the true sperm concentration will be 250 x 10^4 / ml or 2.5 x 10^6 / ml. There should therefore be 2,500 sperm per µl and you should aim to apply ~ 25,000 (10 µl) – 50,000 (20 µl) sperm to the assay slide (see from section 9 onward).

8. In the event that the sample is either too dense or too sparse to read accurately, dilute or concentrate the sample accordingly to bring the sample to a final density that allows straightforward reading.

Optimising sperm loading on to slides. Regardless of the method chosen to prepare slides, you should aim for microscopic fields at x40 magnification with 25 - 50 sperm visible per field. You may need to obtain at least two fields per sample to image a minimum of 50 sperm. The sperm concentration suggested here (~10^6 per ml or 1000 per µl) is based on empirical evidence for optimal sample processing on the imaging platform at Leeds but is for guidance only.

9. Adjust sperm concentration to ~10^6 per ml by adding HBS. If the concentration is too high but it is possible to adjust within the 1.8 ml volume of the sample vessel, then add the required volume. Otherwise, resuspend and remove the required volume from the sample that when *added* to a waiting volume of HBS, will bring the concentration close to 10^6 per ml in 250 µl total volume. If the sample sperm concentration is below 10^6 per ml, re-centrifuge the sample and after discarding the supernatant, re-suspendthe pellet in a smaller volume with HBS calculated to give 10^6 per ml. If it is not possible to obtain this concentration because of very low sperm counts, centrifuge and resuspend in the smallest volume possible that permist the spreading of ~10,000 sperm on either side of a coated glass slide (see point 8).
10. Add 10 µl of the suspension (~10,000 sperm) to either side of a pre-coated glass side (coated side) and spread manually using the pipette tip to a diameter of up to 0.5 cm. If the concentration is slightly lower than 10^6, adjust the spreading volume accordingly. A cytospin (section 10) if available can also be used as an alternative to manual spreading.
11. Place slides on clean blotting paper and allow to air dry for at least 60 min. They can be stored at this point in covered slide containers at 4°C for later use.
12. If a cytospin is used, add a sufficient volume of a fully re-suspended sample containing 10,000 sperm to 1.5 ml of HBS. Place a filter between the slide (coated side) and loading vessel, clamp together and centrifuge for 10 min. Uncouple the vessel and place slide on filter paper as above.

Checking slides for sample processing optimisation.

Before any downstream assay, it is advisable to check your slides and ensure there are sufficient sperm available for analyses. Use the SpermBlue stain for this purpose. Please follow the associated Microptic staining protocol (Appendix 1).

Sample Imaging

Slides should be examined with the same microscope used for downstream analyses.

1. Place slide on microscope stage.
2. Switch on visible light source and locate the sample on one side of the slide.
3. Adjust condenser/iris to give optimal visibility and then switch to x40, increasing brightness as required.
4. Can you count 25 sperm in the field of view?
5. If you can see 25-50 sperm in the field, then your processing has been successful.
6. Switch to other side of the slide and repeat.
7. You will need to visualise at least 50 sperm sperm per sample and preferably 100, so the number of fields required for assay imaging depends on the number of sperm

visible in any individual field. You should be able to use either side of the slide for this purpose.

Recording Images

Leeds uses SmartCapture (see SoP xxx) imaging software running on an Apple iMac. Images are captured from a Zeis Axiomat microscope fitted with epifluoresence and visible light optics and capable of capturing images at x100, x250, x 400 and x1000.

1. Prepare microscope for visible light imaging and launch SmartCapture.
2. Select the option Brightfield (SpermBlue) from the list of choices available (see SoP xxx).
3. Ensure camera and electronic colour filter block are ON and that the filter block is fully engaged in the block housing located in the microscope's C-Mount.
4. Focus on sample at x400 and continue to SoP xxx with reference to the 'SpermBlue' protocol.
5. When you are finished, please remember to turn the camera, colour filter and microscope light source to 'OFF'.
6. Log out of the user account on the iMac.

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Appendix 1: SpermBlue Staining Procedure

KIT FOR SPERM MORPHOLOGY ASSESSMENT IN HUMAN AND ANIMAL SPECIES.

Background:

The stain has been developed to stain all components of sperm (acrosome, head, midpiece, principle piece of tail and end piece) differentially in different intensities of blue. The staining procedure is very simple and only involves two main steps, fixing in one medium and staining in a second medium. It works equally well for smears of "raw" semen as well as swimup/Percoll/PureSperm gradient preparations, using most tissue culture media.

Please note that only fixed/dead sperm to be stained. Not for use with live unfixed cells in any procedure such as in tissue culture.

Contents of SpermBlue®:

All SpermBlue¨ packages contain bottles/staining trays with equal volumes of fixative (clear or transparent solution marked Fix) and a dark blue stain (dark blue staining solution marked Stain).

SpermBlue¨ is packaged in two bottles, individually containing 250 ml fixative and 250 ml stain. Sufficient to stain 600 sperm smears or more on slides.

It is recommended that the fixing and staining of smears are performed in standardized containers,
e.g. plastic Coplin jars, which could be provided on request.

If fixative and stains are stored at 4°C it will last for at least one year or longer. Room temperature storage (20 – 25¼C) not guaranteed but normally lasts one year. Take note of expiry date.

Staining Procedure:

1. Make duplicate sperm smears using 10µl of semen or 10 to 15µl of swim-up sperm (adapt volume to concentration of sperm) and allow to air dry. If sperm concentration in semen is less than 20 million/ml, use 15µl of semen for smear. Ideal angle of slide which is used to make smear is about 45°. If sperm concentration is low, decrease angle of slide which is used to make smear to about 20¼. A larger volume of sperm will accordingly be dragged behind moving slide resulting in more sperm on slide. Ensure sperm smear is totally dry before next step.

2. Carefully place dried smears vertically into staining tray (Coplin-type jar) containing SpermBlue¨ fixative. Take care to slowly immerse slides into fixative and leave – no agitation, etc. Fix for 10 minutes at 20 to 25°C. Alternatively, place dried smears horizontally down on filter paper (smear up). Use a plastic disposable pipette to put 0.5 to 1ml of clear fixative solution on dried smear, and make sure to cover the whole area of the smear. Leave for 10 min – do not roll from side to side.

3. Carefully remove slides from staining tray and hold it at an angle of 60¼ to 80¼ to drain off excess fixative. If smears were fixed horizontally, let most of fixative gently run off slide onto filter paper. No washing or drying is needed after fixation.

4. Carefully place fixed smears vertically into staining tray containing SpermBlue¨ stain. Take care to slowly immerse slides into stain and leave – no shaking or moving of slide in medium. Stain for 12-15 minutes at 20 to 25°C. Alternatively, place slides horizontally down onto filter paper after fixation. Use a plastic disposable pipette to put 0.45 to 0.5ml of stain

onto fixed sperm smear. Gently roll slide from side to side at regular intervals (once every minute) to ensure that stain is displaced equally across smear surface. Stain for 12-15 minutes.

5. Carefully remove slides from staining tray. If smears were stained horizontally, let most of stain gently run off slide onto filter paper. SLOWLY dip stained slides into distilled water – only one dip, lasting for 3 seconds is required. Immersion technique usually requires only one dip to wash off excess stain, but second dip might be necessary for the “drop-on” technique. Slides need to be very gently dipped in distilled water, preventing too many sperm from being lost during this washing step. Remove slides from water very slowly and leave in an upright position (at about 70¼ angle). Let all fluid drain from slide until air dry.

6. Mount slide with DPX or equivalent synthetic medium for making permanent slides. When the mounting medium is dry view under oil immersion x1000 for human sperm.

7. If nuclear staining is not intense enough, stain the duplicate smear for another three to five minutes and even longer if required.

Alternative fixation procedure:
In some instances 95% ethanol or 100% methanol fixation (coagulant fixation) may be used particularly after washing sperm and to concentrate sperm instead of SpermBlue¨ fixation. Staining procedure after alcohol fixation in SpermBlue¨ remains the same as outlined above.

Important comments:
Initial staining results may suggest either too little staining of some sperm as well as differences in staining intensity on the same slide. Each researcher has to experiment to optimize her/his results in this context. Try and adapt staining times at temperature conditions between 20 and 30°C. Many existing sperm staining techniques rely on “sperm painting” which is not cytologically acceptable. SpermBlue¨ clearly differentiates all sub-divisions of sperm accurately and is particularly good in the identification of the sperm acrosome (van der Horst and Maree, (2009) SpermBlue¨: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis, Biotechnique and Histochemistry: In press).

Examples:
With human and sub-human primate sperm the acrosome stains light blue and the head dark blue. Midpiece stains distinctly dark blue, rest of tail slightly lighter blue and end piece even lighter blue. Suitable for SCA automatic Morphology analysis (Microptic SL, Barcelona, Spain).

In domestic animals such as bull, boar and ram: Acrosome stains dark blue, post acrosomal area and particularly the equatorial zone stains light blue. Midpiece stains darker blue and rest of tail slightly less dark blue.

Safety data sheet for SpermBlue®:

SpermBlue¨ contains toxic components like all cytological stains but is not hazardous. The main active component is a slight skin, oral/nasal irritant and staining should preferably take place in a fume hood. If skin contact has occurred, wash affected area thoroughly with water.

Precautions:

All cytological stains are toxic and have to be handled with care. Always work with gloves

and preferably in a fume cupboard. Only stain when sperm are fixed (dead). Do NOT use for live unfixed cells.

Distributed by:

Microptic SL Viladomat 321, 6° 4a, 08029, Barcelona, Spain Tel. +34 934192910 Fax +34 934199426 www.micropticsl.com

Standard Operating Procedures (SOP) for: Acridine Orange staining of sperm			
SOP Number:	05	Version Number:	1.0
Effective Date:	15-02-2016	Review Date:	15-02-2018

Author:	**David Miller**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**
PCTU-reviewed by (Name/ Position):	

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: To stain sperm samples with Acridine Orange. **Objective:** To prepare sperm samples for staining with Acridine Orange. **Scope:** DNA fragmentation assay.

SOP Text
Introduction to technique Acridine orange (henceforward abbreviated to just AO) is a metachromatic dye that under the right conditions of pH and salt concentration, fluoresces GREEN when interacting with intact, double-stranded DNA and RED when interacting with fragmented, single-stranded DNA. It is the basis of the sperm chromatin structure assay (SCSA) that uses fluorescence activated cell sorting (FACS) to measure DNA fragmentation in thousands of sperm cells. The variation detailed in this SoP uses a slide-based alternative that better accommodates the limiting amount of available sample.

Responsibility
Suitably qualified personal in the research lab trained to perform the acridine orange assay.

Activity	
1.	**Reagents and equipment required** Unless otherwise stated, all working stocks and solutions MUST be made up with Analar grade reagents. i. AO solution ready prepared and supplied by Polysciences, Cat No 24603. ii. Solutions of 0.1M NaOH (500 ml) and 0.1M NaOH (500 ml). These can be stored for up to one month at 4°C. iii. Sperm wash buffer (SWB; Origio). iv. Methanol v. Glacial Acetic Acid vi. Phosphate buffered saline (PBS), pH 7.0 (freshly prepared from tablets or powder (Sigma) vii. Microscope slides, acid washed and coated with poly-L-lysine (Sigma) viii. Copling vessels (for washing slides) ix. Carnoy's reagent (9:1, methanol:acetic acid). Once prepared (500 ml) it

	can be kept for up to one month at 4°C x. Epifluorescence microscope equipped with (x400-600) magnification and either fluorescence optics for blue excitation with green and red emission for sequential colour capture (greyscale camera) OR blue excitation with wide band-pass emission filter for simultaneous capture with a colour camera.
2.	**Sample Processing** All samples arrive frozen in 250 µl aliquots. They should be stored at -80°C prior to processing. i. Rapidly thaw sample out by placing in a 37°C water bath. Mix gently and slowly add 500 µl of pre-warmed (to 37°C) PBS, continuing to mix gently. Pellet sperm by centrifugation at 500g for 10 min at room temperature and remove supernatant. ii. Using a 100 µl Gilson-type pipette, gently resuspend the pellet in 100 µl of PBS held at room temperature and add another 400 µl of PBS, mixing gently. iii. Centrifuge as before and resuspend pellet in 150 µl of PBS. iv. Refer to and follow SoP M1 to optimise sperm density. v. Add 10-20 µl of the suspension (15 µl if concentration is low) to either side of a pre-coated glass side (coated side) and spread manually using the pipette tip to a diameter of up to 0.5 cm. Note a cytospin can also be used. vi. Place slides on clean blotting paper and allow to air dry for at least 60 min. Slides can be stored at this point at 4°C in covered containers for later use. vii. OPTIONAL Wet slides by immersing them in water for 5 min. viii. Transfer slides into 0.1M HCl for 30 sec followed by 0.1M NaoH for 30 sec. ix. Fix sperm by transferring slide to Carnoy's solution for 2 hours at room temp. x. Allow to air dry for at least 60 min (on blotting paper). Slides can be stored at this point in covered slide containers at 4°C for later use. xi. If slides were stored cold, allow to warm up at room temp for 5 min. xii. Prepare a 12µg/ml solution of acridine orange stain by diluting 6 µl of commercial stock (20 mg ml) AO solution from Polysciences, inc. Cat# 24603 to 10 ml of working solution with dd water. This should be freshly prepared. It does not keep well so any unused working reagent should be disposed. xiii. Under subdued lighting, flood slides with 1.0 ml AO for 5 minutes. xiv. Run off AO into a suitable container and place in slide rack (preferably glass). xv. Rinse slides free of AO by immersing rack in a glass staining vessel filled with stirring distilled water for 5 min. xvi. Change the water a minimum of three times (5min each); if after 3 washes, the background is too high, when viewing under *green* fluorescence (see below), increase the number of sequential washes. Allow to air dry for 60 min and add a drop of DPX mountant. Slides can be stored at this point in covered slide containers at 4°C for later use. Protect them from light. Note that alternative mountants, including those with anti-fade reagents are not recommended.

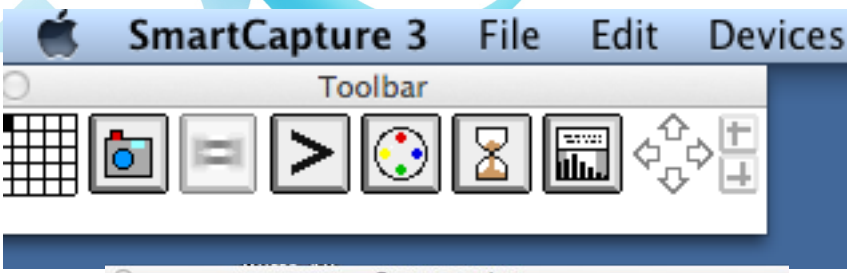
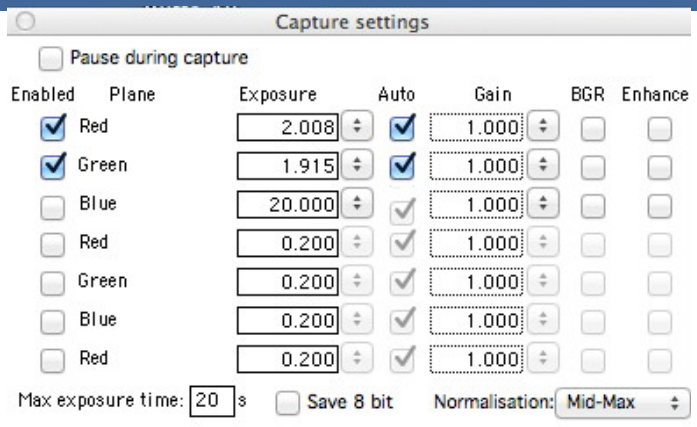
3.	**Sample Imaging and Analysis** (see separate SoP for analogue and digital quantitation).
4.	Reference: Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. J Androl. 2001 Jan-Feb;22(1):45-53. PubMed PMID: 11191087. The SoP is based on the *TAO Method* reported by Tejada et al. Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril. 1984 Jul;42(1):87-91. PubMed PMID: 6724015.

Standard Operating Procedures (SOP) for: Standard Operating Procedures (SOP) for: Image capture and quantitation of acridine orange assay.			
SOP Number:	06	Version Number:	3
Effective Date:	15-02-2016	Review Date:	15-02-2018
Authors:	**David Miller**, **Alex Hargreaves**		
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**		
PCTU-reviewed by (Name/ Position):			
Authorisation:			
Name / Position	**David Miller – HABSelect Chief Investigator**		
Signature			
Date			

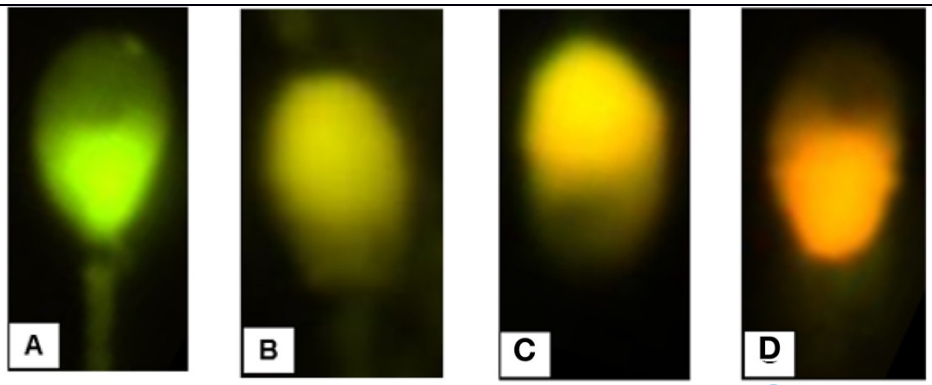
Purpose and Objective:
Purpose: To image and measure levels of sperm DNA fragmentation using Acridine Orange staining. **Objective:** To provide robust measures of red and green fluorescence, the ratio of which, indicates relative DNA fragmentation in each sperm examined. A subjective manual assessment will be made for audit purposes, followed by an objective automated analysis for high throughput. **Scope:** To provide a measure on at least 50 sperm nuclei in each sample.

SOP Text
Introduction to the technique: See SOP04 for prior sample processing and SOP05 for staining steps in the AO assay. The Leeds imaging platform uses a system of 'simultaneous' green and red imaging, recording with an ~450nm blue excitation filter coupled to a wide band-pass emission filter allowing wavelengths of 500nm and above to pass through the *same* excitation/emission block. Emitted green and red light is sequentially captured using the same in-line filters employed for visible light capture (see SOP M2). Alternatively, conventional epifluorescence optics for green and red fluorescence using independent excitation/emission blocks can be used. In either case, a cooled (Hamamatsu) high definition 16 bit greyscale camera is used for image capture. Leeds uses SmartCapture imaging software running on an Apple iMac. Images are collected from a Zeiss Axiomat microscope fitted with epifluorescence and bright field optics and capable of capturing images at x10(0), x25(0), x 40(0) and x100(0) (oil) magnification.

Responsibility
Suitably qualified personal in the research lab trained to perform the acridine orange assay.

Activity	
1.	**Setting up for imaging** **Assay Calibration**. You will already have prepared slides from a fresh sperm sample exposed to H_2O_2 (positive control) or processed without exposure (negative control). This is to enable you to calibrate the capture software. You will already have checked under the microscope that the positive control sample fluoresces dominantly in the red and the negative control, dominantly in the green (see SoP NN). Control slides cannot be re-used for this purpose and should be replaced with fresh controls if the assay needs recalibration. However, the settings obtained should be good for many downstream analyses. i. The UV light on the microscope should be switched on at least 20 min before use. Ensure all objective lenses and clean and free of residual oil beforehand by cleaning with 70% ethanol and allowing to air dry. ii. After switching on the UV light, switch on the camera and colour filter block. iii. Ensure the colour filter block is in place in the microscope's C-mount. iv. Ensure that no light can pass through the microscope by flipping the barrier lever to the 'on' position. v. Launch SmartCapture and select the AO-HABSelect setting from the dialogue box that appears on launch. vi. Ensure block 2 (wide band pass emission filter) is in place in the rotating block holder. vii. Place the positive control slide on microscope stage and select x40 objective. viii. Flip the barrier lever to the 'off' position and adjust the focus so that you can see sperm fluorescing. ix. Activate SmartCapture live imaging (the **>** icon in the figure below). Ensure the image is correctly focused before continuing. Avoid exposing the sample to excessive UV light by flipping and keeping the barrier lever 'on' and only to 'off' when capturing an image.   x. In the SmartCapture image capture settings window, set the exposure to 'automatic' and ensure 'Save 8-bit' is UNCHECKED. Also ensure that only 'green' and 'red' channels are checked on. Blue should be off. xi. Flip barrier lever to 'off' and capture image (click the camera icon in the figure above).

	xii. Viewing through the microscope, locate another suitable field and then refocus for live imaging in SmartCapture (click the **>** icon). Capture image using the same settings as before (click camera icon). Ideally, you should capture at least two fields. xiii. Take a note of the red (positive) channel exposure time (exposure boxes in figure above). xiv. Repeat steps vii – xiii with the non $(H_2O_2)_{exposed}$ (negative control) sample, again noting down the red and green exposure times. The red and green exposure times should be shortest for the positive and negative control sperm, respectively. The reverse will be the case for the longest exposures. If your readings accord with this, then the capture settings have been optimised for both channels. xv. It is now very important to UNCHECK automatic exposure for both red and green channels and type in the SHORTEST exposure times you noted down for each channel. SmartCapture is now calibrated for sample readings. These 'fixed' exposure settings should be good for many downstream analyses, provided that all sample processing steps are identical. *If it is not possible to undertake calibration as above, samples can be imaged using the **same** settings for both channels (auto exposure must be **OFF** and the 8 bit capture unchecked). However, the optimal exposure time (not under or overexposed for each channel) **MUST** be empirically determined.*
2.	**Sample readings** i. Place sample slides on the stage, focus and find fields you can use for image capture. ii. Ensure that automatic exposure is UNCHECKED and that you have typed in the shortest exposure values in the green and red channel boxes, respectively. Also make sure that 'save 8 bit' is UNCHECKED. iii. Capture as before, following steps in section 5.
3.	**Image quantitation.** There are two methods that can be used for image quantitation: subjective and objective. Higher magnification (x1000) may be necessary for the most accurate objective results, but reasonable accuracy can still be obtained for x400 – x600 if the larger number of imaged fields required at x1000 proves unworkable. 1. A **subjective** analogue measure is possible, where sperm are given a score of +++ for all green and −−− for all red fluorescence. Anything in between (yellow) is designated ++− or +−− depending on whether the colour tends towards green or towards red. The scores should be taken on the image, NOT on the microscopic observation and can be done at any time after image capture. The operator should note the number of sperm in each of the three categories and then express them as a % of the total number of sperm assessed. For semi-objective quantitation, two trained observers should undertake this task and their scores should vary by no more than 10%. In the image above, A is +++, B is ++-, C is ++- and D is ---. If pictures are being exported for assessment on a different computer, ensure they are correctly marked and Export as RGB TIF files.



2. Image J

For more **objective** quantitative analysis, Image J (or Fiji) is used. This software measures the arbitrary greyscale levels stored in the two-channel image comprising orange- and green- emission filtered exposures of the same field. The typical image size is 1300 x 1000 pixels, or at 40x magnification, a field of 250 x 200 microns.

The first step is to use an AppleScript macro (Windows version not available) that converts nested folders of the SmartCapture 3 Filmstrips to two-channel TIFFs. The macro also collates the exposure times for each channel to a text file:

`SC3_AppleScript_exposures_batch_FINAL.scpt`

Running a second Applescript file afterward may help to keep conformity in the filename syntax:

`AO_renamerscript.scpt`

The pipeline described below is available as an ImageJ macro file:

`ImageJ_AO_16bit_final2.ijm`

This macro targets nested folders of SmartCapture3 TIFF as input, then for each image, performs preprocessing and segmentation steps. It then calculates and stores the mean and standard deviation of the pixel values for each sperm segment in both green and orange frames.

i. Preprocessing: The nested folder for input images is selected with a specific syntax based on export of TIFFs from SmartCapture. The background is first **subtracted** using a **rolling-ball algorithm** (radius 50 pixels or 8 µm) to tare the 'zero' pixel value to foreground contributions only.

ii. Segmentation: Each individual nucleus must then be segmented so that measurements can be recorded by individual sperm rather than by field. Taking a **maximum-value projection** of the image and **thresholding** above some constant pixel brightness (220/255 in 8-bit, upper 14% of range) gives a binary mask from only bright foreground objects, excluding fewer than 0.5% of sperm cells. The closed islands in the mask are split into individual cells by **watershed** algorithm. To eliminate debris and cells other than sperm, the resulting set of objects is **filtered by area** (50 – 1000 sq. pixels, or 1.3 – 26 μm^2) and **circularity** (4π x area/perimeter^2 = 0.60-0.96).

iii. Uncorrected 'acquired colour ratio': The **mean and standard deviation** of the distribution of pixel values within each remaining object are calculated **for orange** and **for**

green frames. The **ratio of these** mean pixel values then forms a green/orange 'acquired colour ratio' for each nucleus:

$$ACR = \frac{\text{(mean green pixel value)}}{\text{(mean orange pixel value)}}$$

As the uncorrected ACR is confounded by varying exposure times in green and orange, it does not depend only on the true colour of the sperm. It is skewed by the relative brightness of sperm cells to other objects in the same field.

Iii. Corrected 'intensity colour ratio': The true light intensity emitted from each object is proportional to the *rate* at which pixel brightness accumulates over an exposure; that is to say, the **mean pixel value** *divided by* the corresponding **exposure time**. The ratio of true intensities is:

$$ICR = \frac{\text{(mean green pixel value / green exposure time)}}{\text{(mean orange pixel value / orange exposure time)}}$$

$$ICR = ACR \times \frac{\text{(orange exposure time)}}{\text{(green exposure time)}}$$

The exposure times are recorded automatically by SmartCapture at the point of acquisition. The correction factor applies in the same way for both fixed and automatic exposure settings. For less damaged (green) sperm, ICR > 1, and ICR < 1 for an orange sperm.

For each sample, the ICR distribution over multiple sperm has a mean and standard deviation. These are reported as is. However, to compare with other measures of DNA fragmentation the ICR must be converted to DFI:

$$DFI = 1 / (1 + ICR)$$

A final metric, 'damaged % by AO', is defined as the fraction of sperm whose DFI lies above an empirical threshold of 0.65 as calibrated in Birmingham. This is a binary categorical measure of the distribution rather than a linear average over all sperm. The metrics reported are all numeric and dimensionless.

For 8-bit uncorrected AO: ACR mean, ACR standard deviation.
For 16-bit uncorrected AO: ACR mean, ACR standard deviation.
For 16-bit corrected AO: ICR mean, ICR standard deviation, number of sperm analysed, Damaged %.

The following Python script:

`AOexcelcollator_TIFFexposures.py`

collates the ImageJ results and the exposure times from the nested sample folders to a summary Excel spreadsheet, in which the ICR and DFI are calculated for each sperm.

	The mean and standard deviations of these per-sperm metrics are then calculated and reported for each sample with sufficient sperm n > 50.

Version	Reason for Change	Date Approved
1.0	Original	
2.0	Minor optimisation and refinement	
3.0	ImageJ analysis pipelines and reported metrics (Alex Hargreaves)	

List of appendices

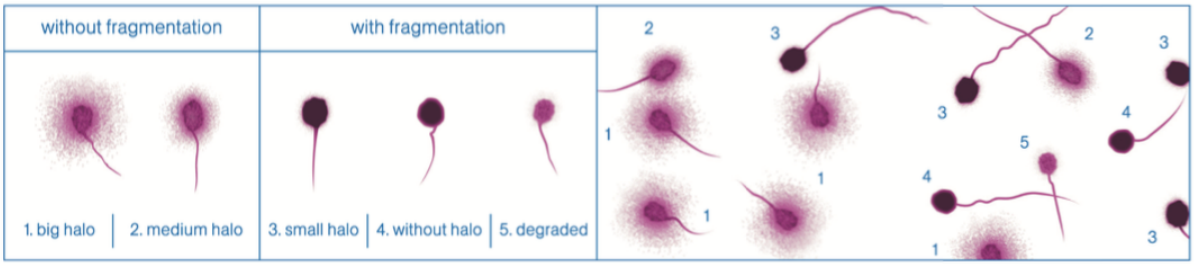
Appendix	Appendix name
A	

Standard Operating Procedures (SOP) for: HALO assay.			
SOP Number:	**07**	Version Number:	**1.0**
Effective Date:	**15-02-2016**	Review Date:	**15-02-2018**
Author:	**David Miller**		
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**		
PCTU-reviewed by (Name/ Position):			
Authorisation:			
Name / Position	**David Miller – HABSelect Chief Investigator**		
Signature			
Date			

Purpose and Objective:
Purpose: To measure sperm DNA fragmentation. **Objective:** The assay uses a proprietary HALO assay to measure the relative intactness and hence quality of sperm chromatin. This is achieved by measuring the extent of halos surrounding sperm nuclei following their treatment with proprietary reagents supplied by the company (Halotech). It is based on the Sperm Chromatin Dispersion (SCD) test (Fernández et al, 2003). **Scope:** To allow the rapid assessment of at least 50 sperm nuclei in each sample.

SOP Text
Introduction to technique Intact unfixed spermatozoa are immersed in an inert agarose microgel on a pre-treated glass slide. An initial acid treatment denatures DNA in those sperm with fragmented DNA. Following this, the (supplied) lysing solution removes most of the nuclear proteins and in the absence of massive DNA breakage, produces nucleoids with large halos of spreading DNA loops emerging from the central core. The nucleoid from spermatozoa with fragmented DNA, however do not show a dispersion halo or the halo is minimal.

Responsibility
Suitably qualified personal in the research lab trained to perform HALO assay.

Activity	
1.	**Reagents and equipment required** 1. See instruction and protocol sheet supplied by the manufacturer (Appendix 1). Do NOT follow the general sperm preparation SoP. Brightfield microscope equipped with x40-60 objective.
2.	**Sample Processing** Follow instruction and protocol sheet supplied by the manufacturer. (Appendix 1). See below for guidance images of Wright-stained sperm processed through the supplied SCD protocol.
3.	**Sample Imaging.** *Always refer to supplied instructions for the setting up of the Leica DM RBE microscope before using it*. 1. Place slide on microscope stage. 2. Switch on visible light source. 3. Using a blank slide and the x40 (or x60) objective, adjust condenser, aperture and field diaphragms to give optimal visibility. 4. Place sample slide on stage and select the x10 objective initially. 5. You will need to visualise at least 50 sperm per sample and preferably 100, so the number of fields required for assay imaging depends on the number of sperm visible in any individual field. Examples of (Wright stained) sperm halos are shown in the figure below. 
4.	**Recording Images and image quantitation** There are two methods that can be used for image quantitation. 1. A subjective, analogue measure is possible, where sperm are given a score of +++ for a large halo, ++- for a medium halo, +-- for a small halo and --- for no halo or degraded sperm. The scores should be taken on the captured image, NOT on the microscopic observation and can be done at any time after image capture. The operator should note the number of sperm in each of the four

	categories and then express them as a % of the total number of sperm assessed. For semi-objective quantitation, two trained observers should undertake this task and their scores should vary by no more than 10%. In the image above, 1 is +++ (not fragmented), 2 is ++- (mildly fragmented), 3 is +-- (fragmented) and 4, 5 is --- (heavily fragmented).
6.	**Image J** See separate SoP05 for digital quantitation of halo dimensions using Image J.
7.	**References** 1. Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, et al. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril. 2005 Oct;84(4):833-42. PubMed PMID: 16213830. Epub 2005/10/11. eng. 2. Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl. 2003 Jan-Feb;24(1):59-66. PubMed PMID: 12514084. Epub 2003/01/07. eng

Version	Reason for Change	Date Approved
1.0		
2.0		

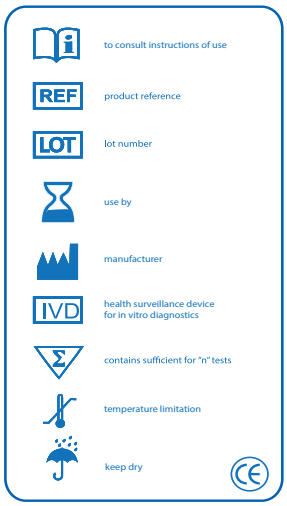
List of appendices

Appendix	Appendix name
A	Manufacturer's Protocol Sheet for Halosperm®

halotech®

halosperm®

Kit REF HT-HS10 for 10 determinations



Application

halosperm® kit from Halotech DNA, S.L. is a simple test that allows assessment of sperm DNA fragmentation in humans.

Principle of the method:
The method is based on the Sperm Chromatin Dispersion (SCD) test (Fernández et al., J. Androl 24: 59-66, 2003; Fertil Steril 84: 833-842, 2005). Intact unfixed spermatozoa (fresh, frozen/unthawed, diluted samples) are immersed in an inert agarose microgel on a pretreated slide. An initial acid treatment denatures DNA in those sperm cells with fragmented DNA. Following this, the lysing solution removes most of the nuclear proteins, and in the absence of massive DNA breakage produces nucleoids with large halos of spreading DNA loops, emerging from a central core. However, the nucleoids from spermatozoa with fragmented DNA either do not show a dispersion halo or the halo is minimal.

Description of kit reagents:
Each kit is sufficient for 10 tests:

- SCS (Super Coated Slides). 10 units
- ACS (Agarose Cell Support). 10 epp.
- DA: 1 Tube with Acid Denaturation Solution Tube of 1 ml.
- LS (Lysis Solution). Bottle of 100 ml

Material and equipment required not provided with the kit:
Bright field or fluorescence microscope. 4°C fridge. 90-100°C and 37°C incubation bath(s). Plastic gloves. Glass slide covers (18 x 18 mm or 22 x 22 mm). Micropipettes. Trays for horizontal incubations. Distilled water. Ethanol 70%, 90%, 100%. Microwave oven and fume hood.

Recommended solution for microscopy visualization:

- Bright field microscopy: Diff-Quik stain (several trademarks) or Wright solution (Merck 1.01383.0500).
- Fluorescence microscopy: Fluorochromes for DNA staining.
- Phosphate buffer solution pH 6.88 (Merck 1.07294.1000).
- Mounting medium: Eukitt® (Panreac 253681).

Specimen:
Samples should be collected in clean containers and the assay should be done as soon as possible. Cryopreserved and freezed samples at -20°C could be also processed. In this case, the sample will be totally thawed, and brought to room temperature before testing, which should be done right after thaw out.

Instructions for use

1. Set the lysis solution (LS) at room temperature (22°C).
2. Dilute the semen sample in culture medium or sperm extender or PBS to a concentration of 5-10 million per milliliter. Be careful that solvent is not extremely dense.
3. Put the agarose eppendorf (ACS) provided through a float; the float should be at the level of the top of the tube. Leave floating in water 5 minutes at 90° -100°C, until the agarose dissolves. Alternatively, the agarose can be melted in a microwave oven.
4. Transfer the agarose eppendorf, with the float, to a temperature controlled water bath maintained at 37°C and leave for 5 minutes until the temperature is even.
5. Add 25 microliters of the semen sample to the agarose eppendorf and mix well. Place the cell suspension from the agarose eppendorf (SCS) on to the treated side of the slide and cover with a glass coverslip, being careful to avoid air bubbles. A drop of 10 or 15 µl for an 18 x 18 mm or 22 x 22 mm coverslip respectively, is recommended. If liquid does not spread till the edge of the coverslip, press gently with the tip of the micropipette.
6. Take care to keep the slide in a horizontal position throughout the entire process.
7. Place the slide on a cold surface (for example, a metal or glass plate pre-cooled at 4°C). Put the cold plate with the slide/s into the fridge at 4°C and leave the sample to gel for 5 minutes.
8. Prepare the denaturant solution (DA). To do this, add 80 microlitres of the contents of the acid denaturation solution (tube labelled DA) to 10 millilitres of distilled water, mix and place in the incubation tray.
9. Remove the slide cover by sliding it off gently. From now on, wear gloves and use slide horizontally with the help of a lancet.
10. Immediately, immerse the slide into the DA solution from step 8 in a horizontal position, leaving it to incubate for 7 minutes.
11. Afterwards, place it in another incubation tray containing 10 ml of tempered LS. Incubate for 25 minutes.
12. Pick the slide up and set up horizontally into a tray containing abundant distilled water in order to wash the lysis solution. Leave to incubate for 5 minutes.
13. Place the slide horizontally into a tray with 70% ethanol (2 minutes), followed by 90% ethanol (2 minutes) and finally, 100% ethanol (2 minutes).
14. Leave to dry at room temperature and stain.
15. After drying, the processed slides may be kept in archive boxes at room temperature, in the dark, for months.

Microscopic visualization

For bright field microscopy, it is recommended two types of stain:

- Diff-Quik stain.Through the following guide: incubate the slide in horizontal position in the Eosin solution(red colour)for 7 minutes. Afterwards, incubate the slide in horizontal position in the Azur B solution (blue colour) for 7 minutes.
- Wright stain, Prepare the Wright solution by mixing it in phosphate buffer (1:1). While keeping the slide in horizontal position, cover the sample with a gentle layer of the dying solution. Leave it for 10-15 min blowing on it from time to time. Remove the dying solution, wash briefly and carefully in tap water and air dry.

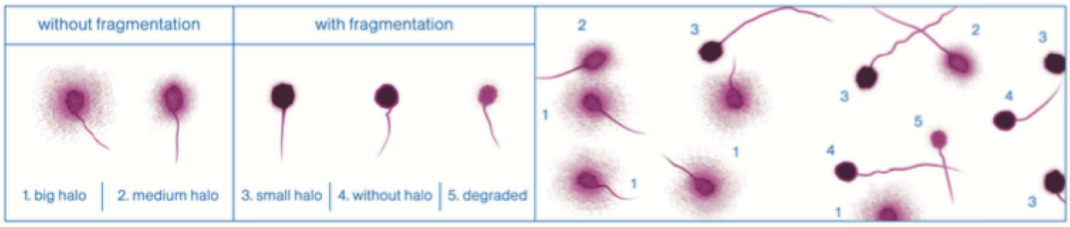
Check colouring level under the microscope. Strong staining is preferred, to clearly discriminate the peripheral border of the halo. If staining results are very weak, especially on the region of chromatin dispersion halos, the slide can be re-stained. If colouration is too strong, the slide can be discoloured by washing gently in tap water, or 10% ethanol if preferred. After air dried, it can be dyed again with reduced colouring exposure time. Once the desired level of colouration is achieved and the slide is perfectly dried, it can be mounted in a permanent mounting medium such as Eukitt®.

Visualization under fluorescence microscopy is also possible using standard DNA directed fluorochromes. 20x and 40x objectives are the optimum for visualization and classification of spermatozoa.

Sperm classification

Removal of sperm nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. The sperm tails remain preserved. For sperm classification we must take into account that sperm DNA fragmentation is a continuous process which produces a series of different halo sizes. Initially, the study of a minimum of 500 spermatozoa per sample is recommended, adopting the criteria of Fernández et al. (Fertil Steril 84: 833-842, 2005). Avoid scoring cells close to the edge of the microgel. Classification:

- **Spermatozoa without dna fragmentation:**
  - **spermatozoa with big halo:** those whose halo width is similar or higher than the minor diameter of the core (Figure 1).
  - **spermatozoa with medium-sized halo:** their halo size is between those with large and with very small halo (Figure 2).
- **Spermatozoa with fragmented dna:**
  - **spermatozoa with small halo:** the halo width is similar or smaller than 1/3 of the minor diameter of the core (Figure 3).
  - **spermatozoa without halo:** (Figure 4).
  - **spermatozoa without halo and degraded:** those that show no halo and present a core irregularly or weakly stained (Figure 5).
  - **"others":** cell nuclei which do not correspond to spermatozoa. One of the morphological characteristics which distinguish them is the absence of tail.



Positive and negative controls

Positive control: sperm cells with halo. Follow the instructions for use, omitting steps 8 and 10.

Negative control: sperm cells without halo. Follow the instructions for use, but omit steps 8 and 10, after step 9 apply a denaturation treatment on the microgel with 10 microliters of DA (undiluted), and gently place a cover slide without pressure, during 5 minutes. Continue the instructions for use from step 11 onwards.

Limitations

This test provides presumptive quantitative information of DNA fragmentation in spermatozoa. This parameter should be analysed by a specialist. The results should be evaluated taking into account all clinical and laboratory findings related to the same sample.

Safety and the environment

Attention! Slide processing must be performed under fume hood.

Avoid inhalation and contact with the solutions supplied. The acid solution (DA) contains Hydrochloric acid, and the lysing solution (LS) contains Dithiothreitol and Triton X-100. Consult specifications supplied by manufacturers.

Do not release the products used into the environment. Follow center guidelines for the storage and disposal of toxic substances.
Biological samples must be handled as potentially infectious.

Precautions

For professional use only.

1. All patient samples and reagents should be treated as potentially infectious and the user must wear protective gloves, eye protection and laboratory coats when performing the test.
2. The test should be discarded in a proper biohazard container after testing.
3. Do not eat, drink or smoke in the area where specimens and kit reagents are handled.
4. Do not use beyond the expiration date which appears on the package label.
5. It is recommended the use of gloves and face mask.

Storage conditions

The kit should be stored at room temperature (2-30ºC). Once open, store LS in the fridge protected from the light. Expire date is printed on the outside of the box.

Halotech DNA, S.L.
C/ Faraday, 7 Parque Cientifico de Madrid / Edificio CLAID / Campus de Cantoblanco / 28049 Madrid, Spain.
Tel. + 34 91 279 69 50 / www.halotechdna.com / info@halotech.es

Patent nº: ES200400163
10 det IUHALOSPERM REV07
Version 07/2012 06 16

Standard Operating Procedures (SOP) for: Aniline blue staining of sperm			
08	08	Version Number:	1.0
Effective Date:	15-02-2016	Review Date:	15-02-2018

Author:	**David Miller**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**
PCTU-reviewed by (Name/ Position):	

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: To stain sperm samples with Aniline Blue. **Objective:** To evaluate sperm chromatin integrity. **Scope:** Chromatin integrity assay.

SOP Text
Introduction to technique The proportion of well-condensed sperm heads (i.e. the quality of the DNA condensation within the sperm head) can be evaluated on smears by the percentage of heads not stained by acidic aniline blue staining. More precisely, Aniline blue binds lysine-rich histones in immature sperm.

Responsibility
Suitably qualified personal in the research lab trained to perform the Aniline Blue assay.

Activity	
1.	**Reagents and equipment required** Unless otherwise stated, all working stocks and solutions MUST be made up with Analar grade reagents. i. Methanol: M/4056/17, Fisher ii. Acetic acid: A/0400/PB17, Fisher iii. PBS tablets: 18912-014, Gibco iv. Ready to use Aniline blue: B8563, Sigma v. Polysine adhesion slides: 631-1349 ThermoScietific vi. Distilled water

	i. Microscope slides, acid washed and coated with poly-L-lysine, Thermo Scientific 2800AMNZ or VWR 631-0107. ii. Cover slips (22x22mm 0.13-0.17mm thick, Fisher MCS22222, SLS 1200-03-14 or Smith Scientific NPS13/2222). iii. Eppendorf tube 1.5ml, Sarstedt 72.690.001. iv. Copling vessels (for washing slides). v. Fixative composed of methanol and acetic acid (3:1). vi. 2.5% (w/v) Aniline Blue in 2% acetic acid (freshly made).
2.	**Sample Processing and staining** All samples arrive frozen in 250 µl aliquots. They should be stored at -80°C prior to processing. i. Rapidly thaw sample out by placing in a 37°C water bath. Measure sample volume when transferring to Eppendorf tubes and slowly add 250 µl PBS, mix it gently and add make the volume up to 1.5 ml using PBS. ii. Centrifugation at 600g for 10 min at room temperature and remove supernatant and keep the pellet. iii. Using a 100 µl Gilson-type pipette, gently resuspend the pellet in 100 µl of PBS and add another 400 µl of PBS, mix it gently and make the volume up to 1.5 ml using PBS. iv. Centrifuge as before and resuspend the pellet in 100 µl of PBS. Measure the final volume to calculate total sperm count in the sample. v. Refer to and follow SoP 01 to optimise sperm density. vi. If the sperm concentration is low pellet sperm as before. Remove the supernatant, leave 10-20µl of the supernatant (PBS) to resuspend the pellet with. Apply 200-300 * 10^3 sperm onto the slide as a drop. vii. Place slides on a clean blotting paper and allow to air dry for at least 60 min. Slides can be stored at this point at 4°C in covered containers for later use. viii. Fix sperm by immersing slides in a solution of Methanol/acetic acid (3:1) for 60 min at room temperature. ix. Allow to air dry for at least 60 min. x. If you have a crease pen, circle the sample. Keep the fixed slides in the fridge if you are not staining that day. xi. Flood the slide with Aniline blue staining solution for 20 mins at room temperature. xii. Wash the slides by dipping in water for 3sec, gently shake or dab the slide. xiii. Repeat step xii. xiv. Leave to air dry for 1 hour on the bench. xv. Mount with DPX using cover slip, leave to set. xvi. Store in the fridge with silica pouches.
3.	**Sample Imaging and Analysis** (see separate SoP 10 for analogue and digital quantitation).
4.	**Reference** -Auger J, Mesbah M, Huber C, Dadoune JP. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between

proven fertile and suspected infertile men. Int J Androl. 1990 Dec;13(6):452-62.

-Sellami, A. et al. 2013. Assessment of chromatin maturity in human spermatozoa: useful aniline blue assay for routine diagnosis of male infertility. *Adv Urol.* **2013**, p578631.
Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril. 1984 Jul;42(1):87-91. PubMed PMID: 6724015.

Standard Operating Procedures (SOP) for: Image J quantitation of captured images (Leeds local version)			
SOP Number:	09	Version Number:	1
Effective Date:	15-02-2016	Review Date:	15-02-2018

Author:	**David Miller**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**
PCTU-reviewed by (Name/ Position):	

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: To provide robust, digital measures of thresholded 'blobs' on captured images. **Objective:** Wholly objective imaging not subject to operator bias. **Scope:** Any downstream analysis reliant on numerical input.

SOP Text
Introduction to the technique: This procedure applies to Image J processing of digitally captured images that will normally be full colour or greyscale and relies on measuring pixel brightness in individual channels (red, green and blue). See SOPs 04 and XX for prior sample processing for AO and aniline blue assays, respectively. The basis of the process is the measurement of pixel 'brightness' in 8 bit or preferable 16 bit greyscale image. Colour images will normally be split into their respective red, green and blue channels prior to conversion of each channel to 16 bit greyscale for this purpose. Uncompressed TIF images are recommended so capture settings or export functions in other SoPs should be set for 16 bit capture (8 bit unchecked in SmartCapture) and exported as uncompressed TIFs.

Responsibility
Suitably qualified personal in the research lab trained to perform the acridine orange assay.

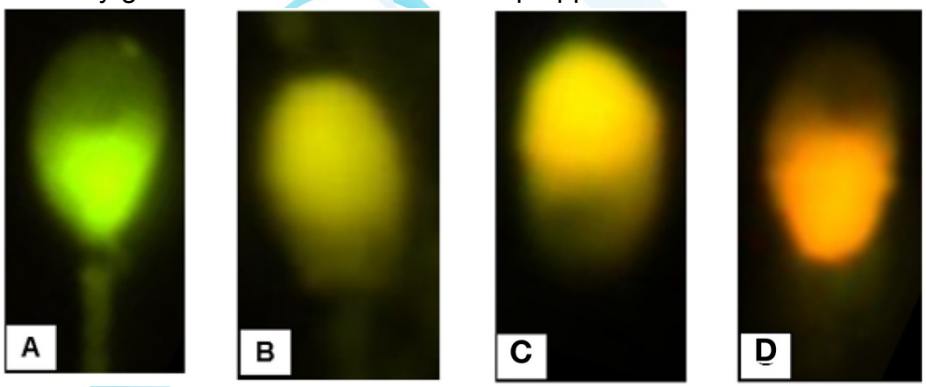
Activity

1. The open source 64 bit **Image J** package is needed for this procedure (check Google). This software runs in a Java environment, which must be installed on the computer workstation where the image analysis is being run. The choice of workstation platform is irrelevant and Windows, Mac and Linux are all supported. Any reasonably modern computer with at least 2Gb of RAM and a good quality colour display can be used. See below for general and specific examples.

   General Instructions for Image J: -

   With **Image J** running:-

   i. Drag and Drop the uncompressed **RGB** TIF or JPG of your choice on to the Image J toolbar or navigate to the file using the menu File → Open → Directory (where file resides) menu choices.
   ii. Select menu Image → Type → and set to 8 bit or 16 bit depending on whether the original is an 8 bit of 16 bit image. The colour image should change to greyscale.
   iii. Select menu Image → Adjust → Threshold. The Threshold dialogue box should open and objects in the window frame should transform from white or grey to red. If they are not red, ensure that the Threshold options are set to 'Default' and 'Red'. Use the upper slider to ensure as much of the object as possible is painted red but without overlapping another nearby object.
   iv. After clicking the 'Apply' button in the Threshold box, objects in the frame should revert to white on a black background.
   v. Select menu Process → Binary →Watershed. This should separate any overlapping objects.
   vi. Select menu Analyze → Analyse Particles. An analyse particles dialogue box should open. Ensure that the Show has 'overlay outlines' and 'Add to Manager' checked. When the OK button is pushed, a new 'ROI Manager dialogue box should open). You can dismiss the two other boxes but **keep the ROI Manager open**. Objects in the threshold window should now be numbered. If they are not, uncheck and recheck the Labels option in the ROI Manager.
   vii. Go back to threshold window and select menu Image → Overlay → Flatten. This ensures that the labelled objects are preserved when you save the image by selecting menu File → Save As → Jpeg to a destination of your choice. Name the file such that it can be associated with the original file image and include 'LUT' in the filename. For example, if the original file that you split into three channels is called 'file_123', name this new file, file_123_LUT. You can now dismiss all open windows including the image window **but keep the ROI Manager open**.
   viii. Reopen the **original** image and select menu → Color → Split Channels. The three colours comprising the original image will open in separate windows. Assuming that an RGB image was opened, the channel's colour will be on each window's title bar.
   ix. Dismiss the window(s) of any channel(s) that you are **not** interested in.
   x. Select one of the open 'color' windows by clicking on it. Go back to the ROI manager and ensure 'Show all' and 'Labels' are checked. The numbers should reappear on each object.
   xi. In menu Analyze → Set Measurements, ensure that area, integrated density and mean gray value are all checked.
   xii. Shift-select all objects in the ROI Manager window and press the 'Measure'

	button. A new 'Results' window will open showing information about the thresholded objects in the channel window. xiii. Select the other 'color' window; there is no need to uncheck and recheck labels in the ROI Manager but the labels may not be visible unless you do so. xiv. Repeat step xii to select all objects and press 'Measure'. The Results window will have all measurements for this channel placed beneath the original output. If a blue channel were available, the process would be repeated and the output added below. The number of rows in the results will therefore depend on the number (n) of objects (O) and the number of channels measured (C) where rows = On x Cn. xv. Closing the window will bring up a 'save results' dialogue that you should accept, naming the file according to the original image identifier. This is important to ensure that the measurements correspond to the image from which they were derived. We suggest that you save the original image, its LUT image and the Excel results in a folder names after the original image.
2.	**Sample reading for red / green including acridine orange and live / dead.** Use the red and green channels. The measured outputs in the case of red-green images will provide green and red pixel intensity measurements that are recorded on the saved spreadsheets. To obtain ratios from these, use the Mean (Gray) values (IntDen or RawIntDen measures give similar results) and divide one by the other. In AO staining, dividing the green signal with its corresponding red signal will give ratios where 1.0 is yellow, >1.0 tends to green and <1.0 tends to red. The opposite will be the case if red is divided by green but the same relationship applies.  Check the results against subjective scoring (+++ to – – –) on the original colour images. Ratios (green/red) for Acridine Orange images would be in the range 1.24-2.42 (A), 0.94-1.28 (B), 0.85-0.94 (C) and 0.44-0.68 (D)
3.	**Sample reading for aniline blue or other blue stains. To be developed**

Version	Reason for Change	Date Approved
1.0		

List of appendices

Appendix	Appendix name
A	

Standard Operating Procedures (SOP) for high-throughput quantitation of Aniline Blue images			
SOP Number:	10	Version Number:	1
Effective Date:	15-02-2016	Review Date:	12-10-2017

Author:	**Alex Hargreaves**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**
PCTU-reviewed by (Name/ Position):	

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:

Purpose: To obtain frequency measures of staining classification for individual sperm in brightfield images of aniline blue histological slides.
Objective: To provide robust measures of aniline blue (AB) staining by imaging under brightfield illumination, including automated classification not subject to operator bias outside training sets.
Scope: To provide a measure on at least 50 sperm nuclei in each sample. Analysis provides database of sperm including comprehensive image metrics and staining classification.

SOP Text

Introduction to the technique:

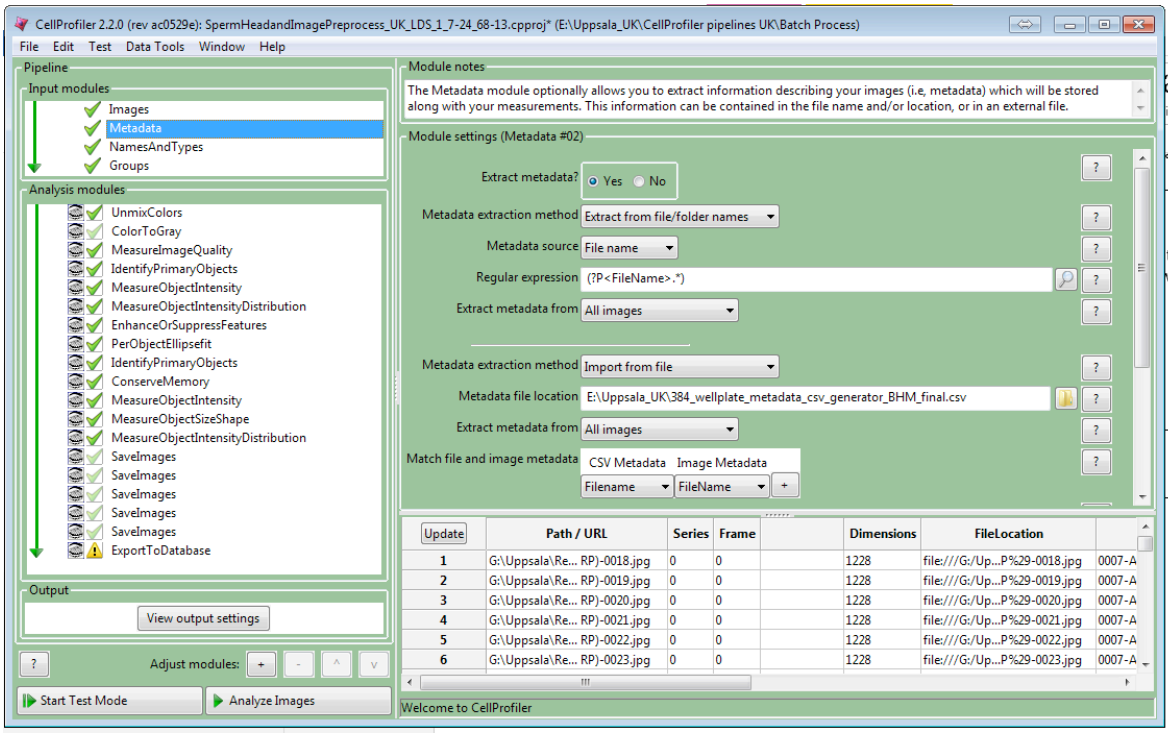
Aniline blue (AB) is a histological stain whose strength indicates poor compaction of DNA in sperm. See SOP04 for prior sample processing and SOP08 for staining. The procedure below uses the suite of CellProfiler open source software to process and analyse digital images obtained through colour brightfield microscopy. The pipeline comprises five distinct parts: cataloguing metadata, image processing, segmentation, training and classification. Processing involves homogenisation of each unmixed image from the catalogued set in turn - presenting a well-defined, uniform input for calculation of a vast number of image brightness and shape characteristics. The segmentation of brightfield images into constituent sperm necessarily depends on object shape as well as intensity information. For a 'training set' of images, these sperm segments are classified according to a moderated manual scoring. This primes an artificial intelligence algorithm to then classify all sperm segments automatically.

Responsibility

Suitably qualified personnel with proficiency in manual aniline blue scoring and CellProfiler and CellProfiler Analyst software.

Activity	
1.	**Image acquisition** The Leeds imaging protocol uses manually-selected fields only, whilst the Birmingham imaging platform improves the speed and breadth of acquisition with slide-scanning functionality. For each setup, the pixel size in the images is calibrated using a graticule. By placing an empty sample (water film under a coverslip), suitable white balance levels and a background subtraction image are set for use with all subsequent acquisitions. A suitable fixed exposure time is used for all samples and all fields, e.g. 40 ms In Leeds, images are acquired on an upright Zeiss Axioplan II at x40 (Zeiss objective, air 0.75NA, 44035001) with a Bayer-filtered colour camera (Toupcam U3 CMOS) under white halogen lamp. Sample slides are placed on the stage, focused upon and suitable fields are acquired independently until 50 – 200 sperm are captured, or the sample presents insufficient sperm and is omitted. In Birmingham, the images are acquired on an inverted Zeiss Axiomat at x40 air with a Bayer-filtered colour camera (QImaging Fast1394) under white halogen lamp. The stage and objective are piezo-motorised (Prior) and controlled by acquisition software (Metamorph) for autofocus and autoscanning functions. Initial inspection occurs as before; however for acquisition, only the first and last fields are selected manually. The sample must be brought manually into sharp focus (within a depth of field of +/- 1 micron at this numerical aperture) for the autofocus to lock on correctly. A scan macro acquires these fields plus every tiled field in between regardless of content. During a scan, the autofocus is updated automatically once every 5 adjacent fields to counter axial drift. The appropriate number of fields in a scan varies with the density of sperm on the slide, but one scan of 5 – 100 fields is usually sufficient for each sample.
2.	**Manual image quantification** A subjective analogue measure is possible, where sperm are given a score of '++' for deep, uniform blue stain, '--' for clear, colour-free cells or with a faint outline, and '+-' for distinct intermediate cases (such as where the tailward part of the nucleus and midpiece are deeply and uniformly stained but the acrosome and nuclear material immediately behind it are not, see Fig 1.).  Fig 1. Exemplary phenotypes for tertiary classification (l-r: weak, medium, strong) in the aniline blue assay. Manual scores should be taken on the normalised colour image, not on the microscopic [illegible] number of sperm in each of the three [illegible] the total number of sperm assessed (which [illegible] trained observers should undertake this task [illegible] should vary by no more than 10% for standardisation. 

3	**Automatic image quantification** Our motivations for developing automated analysis are the following: improved speed, low maintenance, inherent objectivity, reproducibility, and production of records at every step. However, automated approaches often struggle to determine which (of the myriad possible) parameters are important for classification, even in cases where the classification is obvious to the eye. Offsets are to be expected in accuracy and ease of use compared to the manual analysis. Machine learning offers a general approach to image classification starting from a preclassified subset. It solves the problem above, in that it does not require the range of characteristics of each category to be defined explicitly in advance, but defined during training. Once trained, automated classifiers based on machine learning become highly specialised. As such, the classifier will be more accurate if variants are dedicated to each acquisition route (viz. Birmingham and Leeds).
i.	**Software Installation** The open source 64 bit CellProfiler and CellProfiler Analyst packages are needed for this procedure. This software runs in a hybrid Java / Python environment, prerequisites of which must be installed on the computer workstation where the image analysis is being run. Windows XP+ is preferred for compatibility (Bootcamp is supported for Mac). As this procedure is very computationally intensive, RAM of at least 4x the size of each image batch should be used to avoid memory saturation errors (e.g. >16 GB of RAM for 2000 images each of 2 MB). For speed, images should be stored local to the machine, such as on an external hard drive. A typical pipeline takes a few minutes per image / no. cores.
ii.	**Cataloguing and image I/O** CellProfiler requires a metadata file – a spreadsheet of comma-separated values containing a full list of image filenames, dimensions etc. from which it can derive correct import settings.



An example which generates **.csv* files is given in:

```
384_wellplate_metadata_csv_generator_final.xls
```

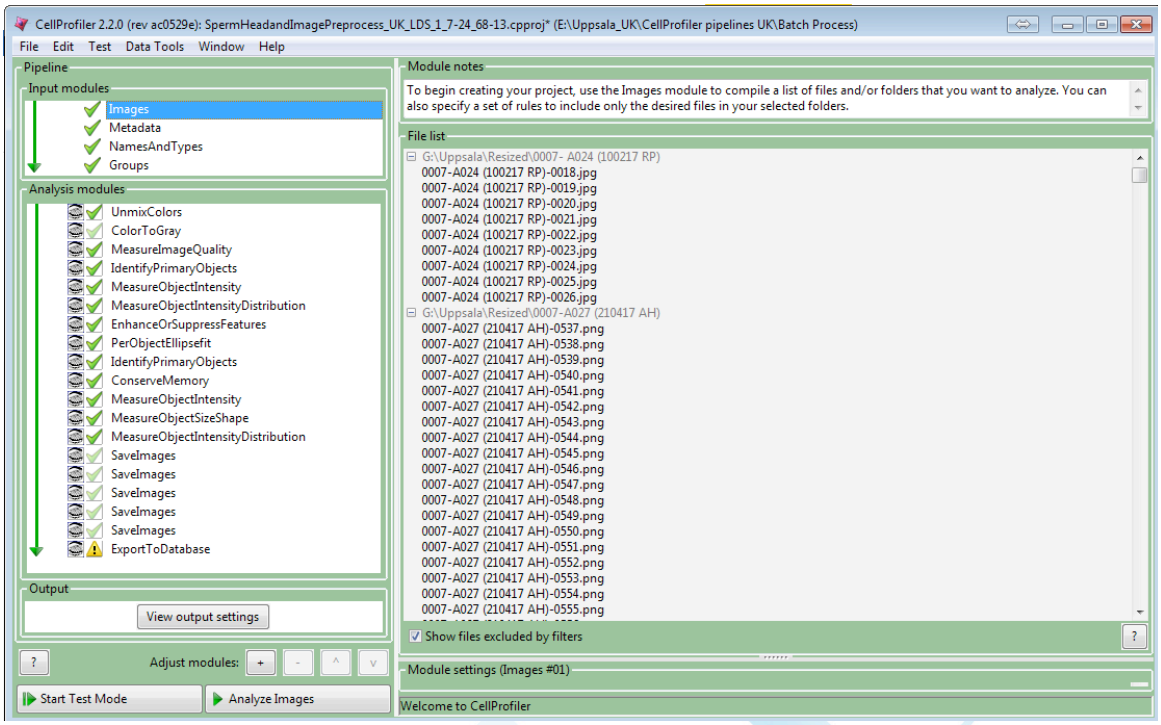
In Windows a list of filenames within the current folder and its subfolders can be extracted to file using the command prompt as follows:

```
> dir -s -b > filelist.txt
```

This can be used to build the metadata file. Including *Plate and Well* fields in the metadata for each image allows later use of the Plate Viewer feature in CellProfiler Analyst (see *iv. Training*). This provides an interactive graphical overview of the results, which can be helpful in identifying subsets in the images, or patterns of poor segmentation.

iii **Processing and segmentation**

The CellProfiler interface includes a preamble for Metadata (see above) and Image import (drag and drop image files from Windows Explorer e.g. **.png, *.tif*).



Finally image processing modules are listed in a custom sequence known as a pipeline. Each module represents a processing step such as resizing, splitting colour channels or creating masks or objects. It may include options such as a choice of algorithms and heuristic parameters that need to be specified for the particular use. These settings are saved as pipeline project files (**.proj*), e.g:

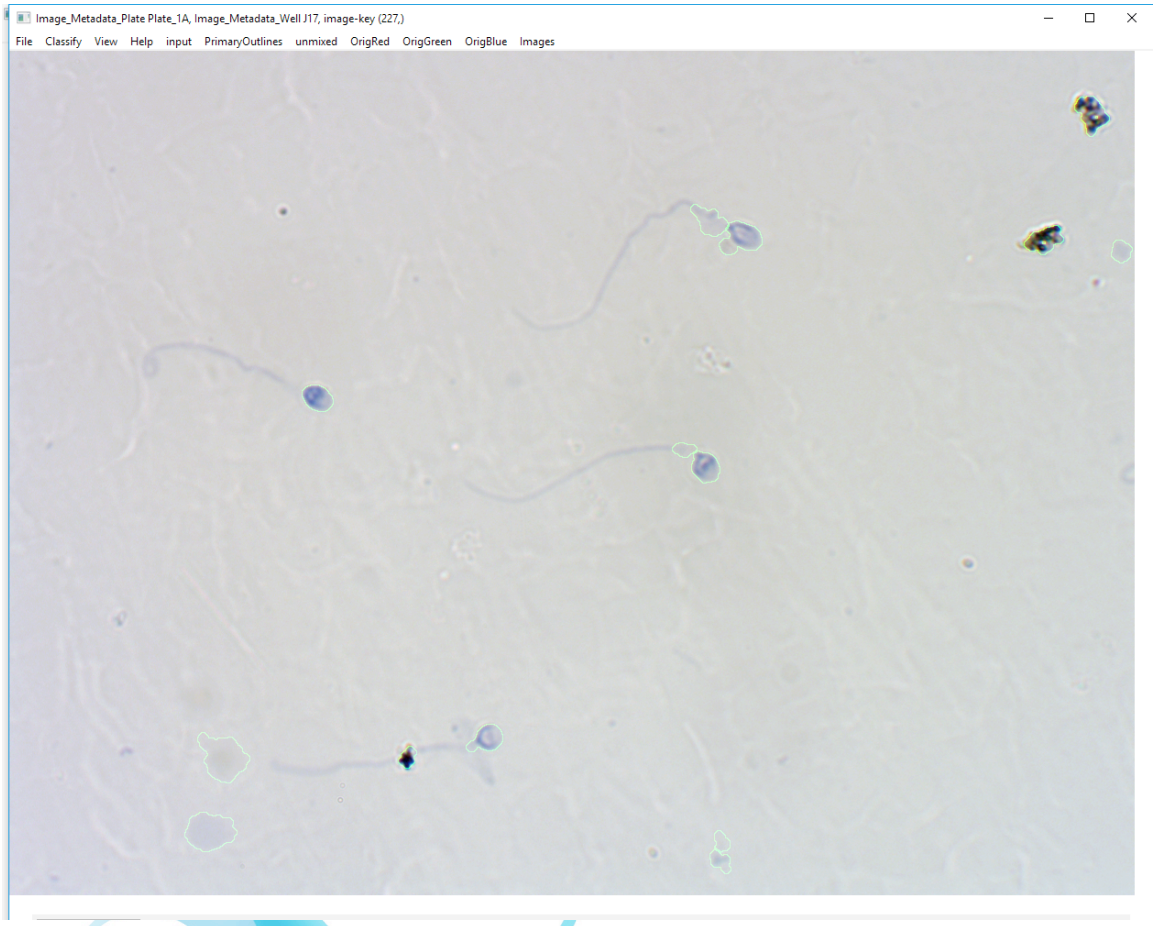
`SpermHeadandImagePreprocess_UK_LDS_4_314_83_314_263.proj`

The Aniline Blue pipeline is as follows:

1. `UnmixColors` – transforms the raw RGB colour image to a greyscale 'unmixed' image using a known colour map for the stain.
2. `ColorToGray` – splits the RGB channels for later visualisation
3. `MeasureImageQuality / IdentifyPrimaryObjects / MeasureObjectIntensity / MeasureObjectIntensityDistribution` – quantify whole-image characteristics such as focal quality, background flatness and whether it contains large clumped areas of sperm.
4. `EnhanceOrSuppressFeatures / PerObjectEllipseFit / IdentifyPrimaryObjects / MeasureObjectIntensity / MeasureObjectIntensityDistribution` – the first is a processing step which reduces the contrast in thin features only. This suppresses the appearance of the sperm tails. The second function is a bespoke algorithm (Petter Ranefall, Uppsala University) which segments non-overlapping elliptical objects of the expected size for sperm heads. The remainder act to measure a large number of arbitrary shape and intensity characteristics of the segments. During training, the classifier will sift through this data to identify significant mathematical differences between categories.
5. `ClearMemory` – erases some intermediate image types from memory to free up space for the heavy calculations on segments.
6. `SaveImages` – saves images and segmented outlines for visualisation
7. `ExportToDatabase` – saves calculated metrics for each segment in each image

to a database.

The segmentation looks similar to this example (thin green lines denote segments). The false positives must be screened out at the classification stage (debris, bottom left patches):



The project file contains the heuristic settings for each of these modules. Alongside the saved images, the completed calculation generates a **.properties* file (for CellProfilerAnalyst) and an associated SQL **.db* database, e.g. the attached:

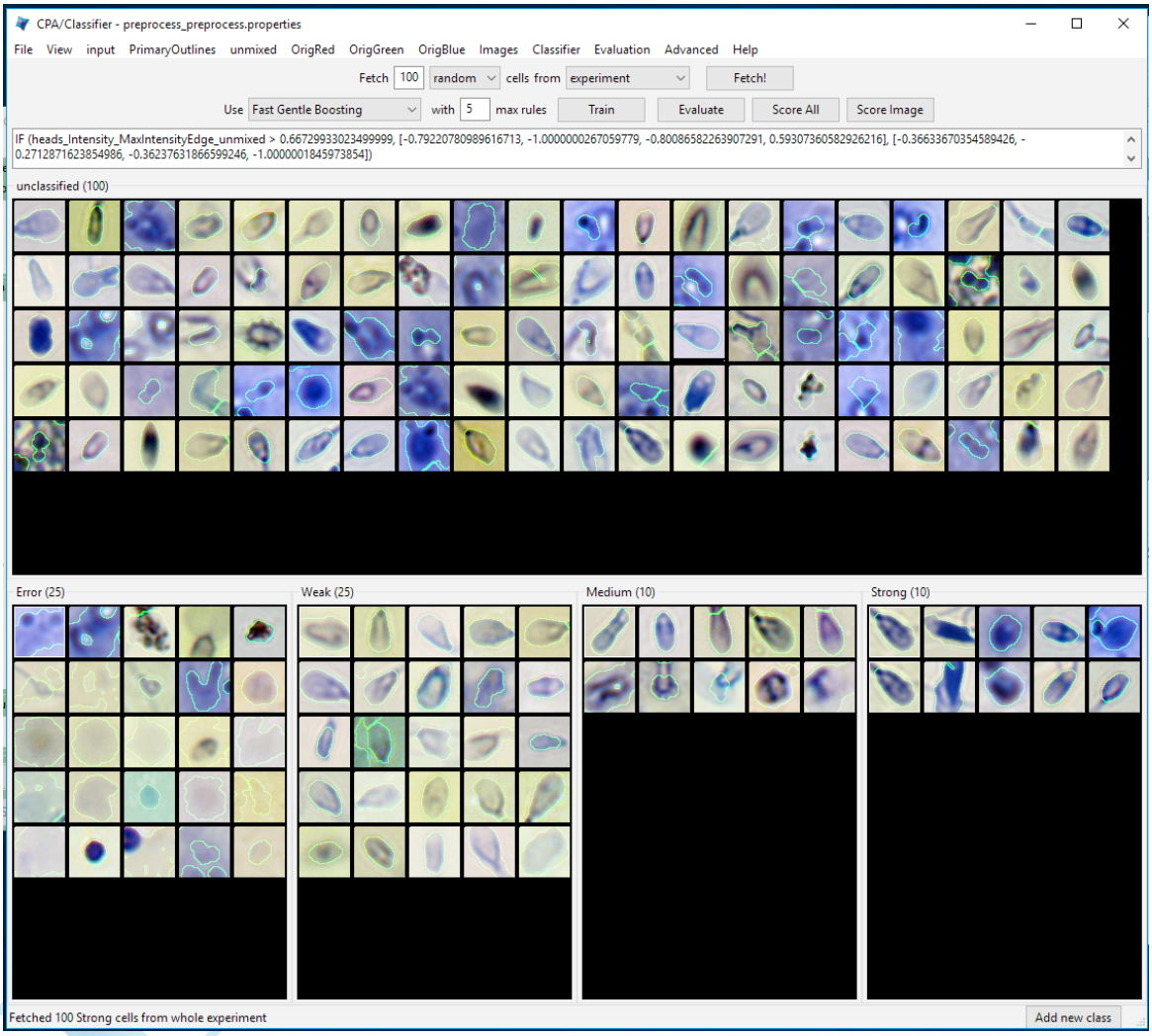
`spermpreprocessLDS_314_83_314_263.properties`
`spermpreprocessLDS_314_83_314_263.db`

iv **Training**

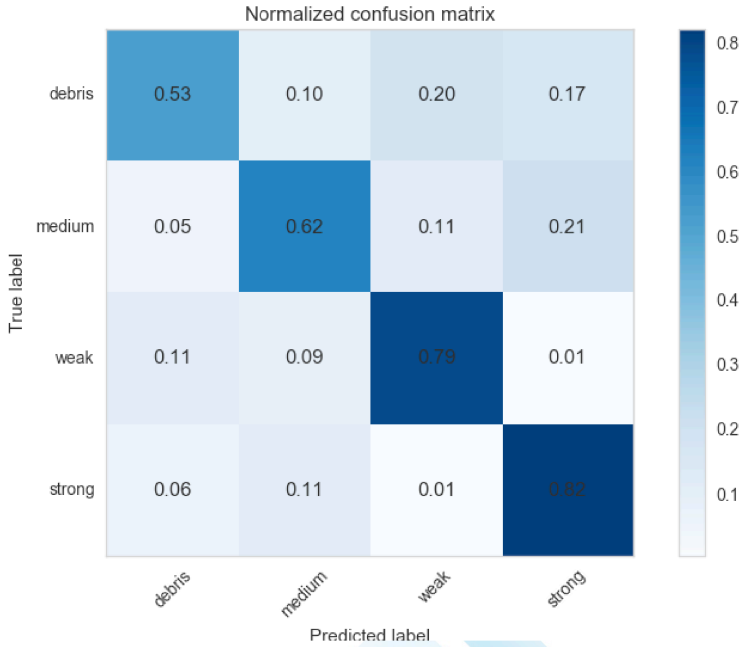
A training set of a recommended 5-10% of the total images are processed and segmented first. It is crucial that these represent the diversity of the full set, so it is best to select most of them randomly plus a few outlier cases by inspection. At least 25% of the training set images must have already been scored manually (see *ii. Manual Scoring*) by at least three authors and standardised according to a number of classifications compatible with the desired model. The manual scoring for sperm under aniline blue stain is typically tertiary: '++', '+-', '--', as described in Section 2. The automated scoring will reflect this.

With the SplitChannel/ColorToGray and SaveImage modules activated in the pipeline, the human-readable image content (colour image with segmentation outlines) can be interrogated in CellProfiler Analyst via the properties file. The Classifier tool allows

categories to be built, corresponding to different degrees of staining, in our case of four classes: '*Weak*'='--', '*Medium*'='+-', '*Strong*'='++', as well as '*Debris/Error*'= false positives in segmentation. Debris typically comprises stained immune cells, glass particles, stray fibres and dark patches of background. This classifier model and training set can be stored in **.model* files and **.csv* spreadsheets respectively.



Segments are gathered (with the 'Fetch' button) and assigned (manual drag and drop) to populate the model categories. Only those segments with a clear phenotype are retained. The classifier algorithm is *FastGentleBoosting* with <15 max rules. These rules are iteratively trained ('Train') to maximise the diagonal elements of the confusion matrix (via 'Evaluate'). Training is very fast, taking just a few seconds but needs refinement iteration by iteration. If the phenotypes are underspecified or too few rules are used, the classifications will appear random unless particular to the cases selected. If they are overspecified, some categories will overlap and the classifier will confuse them.



Once the confusion matrix elements are maximised (a good response is >85%), the optimised rules should be copied from CellProfiler Analyst to a text file, for example:

```
IF (heads_RadialDistribution_ZernikeMagnitude_unmixed_2_0 > 0.291,
    [-0.340, -0.592, -1.000, 0.487], [0.043, 0.040, 0.015, -0.124])
IF (heads_Intensity_UpperQuartileIntensity_unmixed > 0.626,
    [0.157, 0.149, -0.283, 0.040], [-0.264, -0.208, 0.091, -0.381])
IF (heads_Intensity_StdIntensity_unmixed > 0.032,
    [0.013, -0.072, 0.063, 0.000], [-1.0, 0.806, -0.743, -0.114])
IF (heads_AreaShape_MeanRadius > 4.501,
    [-0.011, -0.141, 0.052, 0.047], [0.033, 0.494, -0.267, -0.362])
```

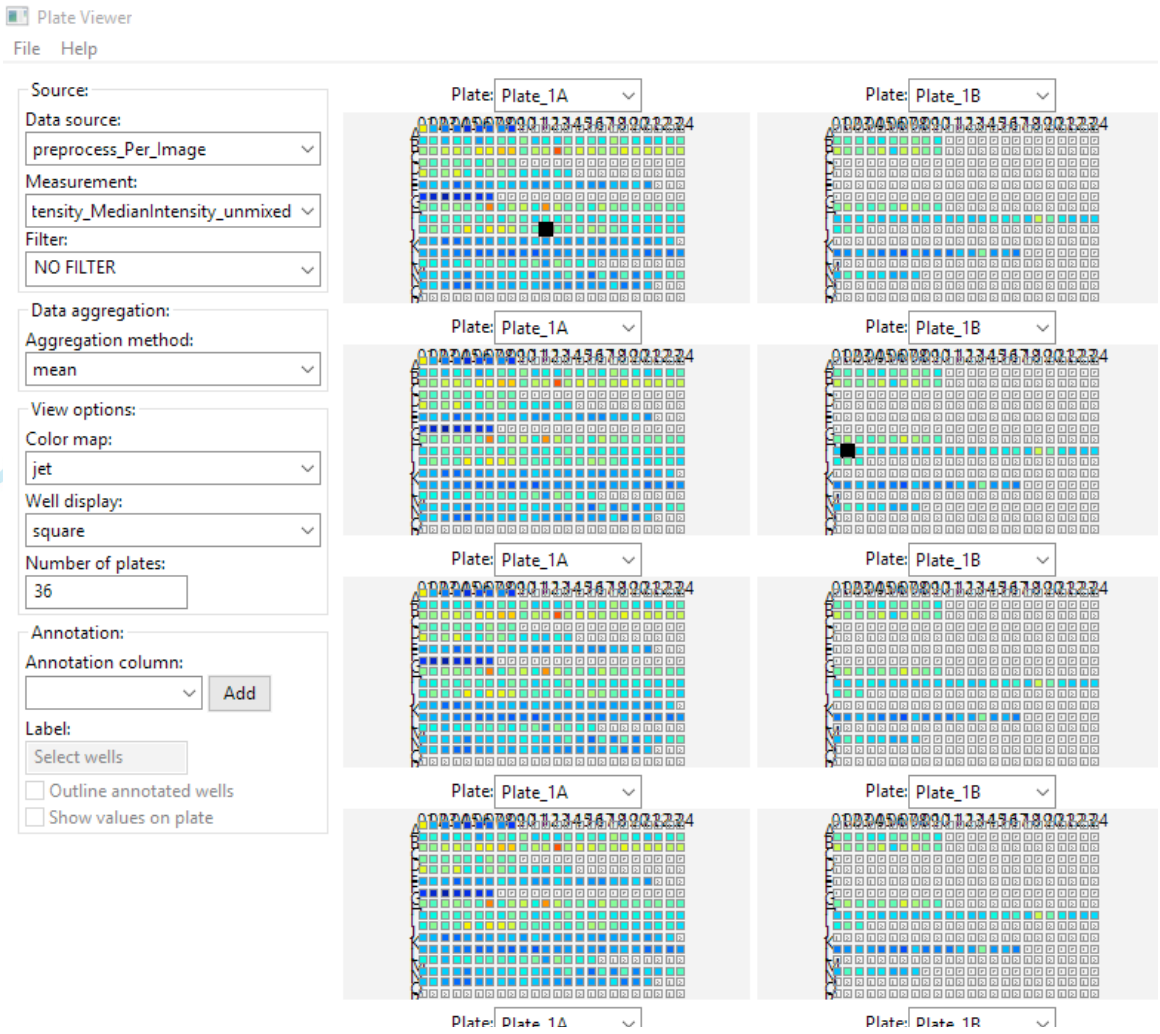
v **Classification**

In CellProfiler, a separate copy of the processing and segmentation pipeline is set up for fast processing, with SplitChannel and SaveImage modules disabled (greyed out) to save memory. Run the truncated analysis pipeline batchwise on the remaining images.

In CellProfiler Analyst, open each properties file in the Classifier and load the classifier model so that the categories appear in the same order. Copy the rules from the text file into the 'Edit Rules' field. Use 'Score All' and export each 'Hit Table' to a spreadsheet. Collate the spreadsheets using Excel or similar. The AB stain is reported here as the percentage of sperm (in strong, medium and weak categories) which fall into the strong and medium categories combined for samples with >50 sperm only:

$$\%\ (++\ \&\ +-) = \frac{100 \times (n\text{Strong} + n\text{Medium})}{n\text{Strong} + n\text{Medium} + n\text{Weak}} \quad \{\text{for } (n\text{Strong} + n\text{Medium} + n\text{Weak}) > 50\}$$

The distributed results for each metric can be interrogated using the Plate Viewer, where each row of spot rep resents a sample, each spot represents a segment or an image, and its colour the value of that metric. It also allows inspection of the raw and segmented images, as well as plotting the multivariate distributions of the selected metrics. This is very useful for auditing the data.



Version	Reason for Change	Date Approved
1.0		

List of appendices

Appendix	Appendix name
A	

Standard Operating Procedures (SOP) for: HALOSperm Assay with Giemsa staining			
SOP Number:	XX	Version Number:	1.0
Effective Date:	15-02-2016	Review Date:	15-02-2018

Author:	**David Miller**
Reviewed by (Name/ Position):	**Jackson Kirkman-Brown - Laboratory Co-Lead**
PCTU-reviewed by (Name/ Position):	

Authorisation:	
Name / Position	**David Miller – HABSelect Chief Investigator**
Signature	
Date	

Purpose and Objective:
Purpose: **Objective:** **Scope:** DNA fragmentation assay.

SOP Text
Introduction to technique Intact unfixed spermatozoa are mixed in an inert agarose micro gel on a pre-treated slide. An initial acid treatment denatures the DNA in the cells with fragmented DNA. Following the denaturation step, the lysis solution removes most of the nuclear proteins. Absence of massive DNA breakage produces nucleoids with large halos of spreading DNA loops, emerging from central core. In contrast, the nucleoids from spermatozoa with fragmented DNA either do not show a dispersion halo or the halo is minimal.

Responsibility
Suitably qualified personal in the research lab trained to perform the HALOSperm assay. All human samples should be treated as potentially hazardous and Personal Protective Equipment (lab coat and gloves) must be worn at all times.

Activity

1. **Reagents and equipment required**

Unless otherwise stated, all working stocks and solutions MUST be made up with Analar grade reagents.

The kit (HT-HS10) contains materials for 10 tests:

SCS (Super coated slides), 10 slides with two sample areas

ACS (Agarose Cell support) 10 Eppendorf tubes with agarose

DA (Denaturing agent) 1ml

LS (Lysis Solution) 100ml

Specific materials and equipment required but not provided with the kit:

Specimen:

The semen sample can be fresh or cryo preserved. If frozen, the sample should be fully thawed, brought to room temperature and processed immediately.

Other:

Distilled water

Ethanol

Incubator for 90-100°C and 37ºC (heat block)

4°C refrigerator

Glass slide covers (22x22mm or 18x18mm)

Portable cold surface (ice pack or pre-cooled metal tray)

Giemsa stain, modified (GS500, Sigma Aldrich)

2. **Sample Processing**

All samples arrive frozen in 250 µl aliquots. They should be stored at -80°C prior to processing. Wash each sample twice with a volume up to 1.5ml of PBS. Centrifugation at 600x g for 10min. Re-suspend the sperm pellet ideally to a concentration of 5-10 x 10^6 sperms per ml. If the sperm count is lower, the volume is kept as small as possible to use the whole sample and the ratio between sperm and agarose be kept 1:4.

HALOSperm Assay

i. Keep the slides horizontally at any time.

ii. Set the Lysis solution (LS) at room temperature.
iii. Place the agarose tubes in the heat block at 90-100°C for 5min until the agarose dissolves.
iv. Transfer the agarose tubes to the 37°C heat block and leave for 5min.
v. From now on, work a sample at the time to avoid solidification of agarose and uneven distribution of sperms on the slide.
vi. Add 25µl or smaller volume of the semen sample to an agarose tube with appropriate volume and mix well by pipetting gently up and down several times without creating bubbles. Return the tube to 37°C.
vii. Add 15µl of the sperm-agarose mix onto the pre-treated side of the SCS slide and cover with a cover slip.
viii. Place the slides on a cold surface and incubate at 4°C for 5min. Note that the incubation time will be longer for the first samples as it is for the last.
ix. Prepare the denaturing solution by adding 80µl of the kit's DA solution into 10ml of distilled water.
x. Remove the cover slip by sliding it gently off to the side.
xi. Immediately, flood the slides with the diluted denaturing solution and incubate at room temperature for 7min. No wash between denaturing and lysis.
xii. Lyse the slides by immersing the slides in 10ml undiluted lysis solution for 25min.
xiii. Wash by submerging the slides in distilled water for 5 min.
xiv. Dehydrate the slides through 70%, 90% and 100% Ethanol, 2min each
xv. Leave to air dry at room temperature
xvi. Continue to Giemsa staining

Giemsa staining

xvii. Make a 1:10 dilution of Giemsa stain in distilled water. Incubate the slides with Giemsa stain for 60min at room temperature.
xviii. Wash the slides gently by dipping in distilled water. Leave to dry at

	room temperature. xix. No cover slips are needed.
3.	**Sample Imaging and Analysis** (see separate SoP for analogue and digital quantitation).
4.	