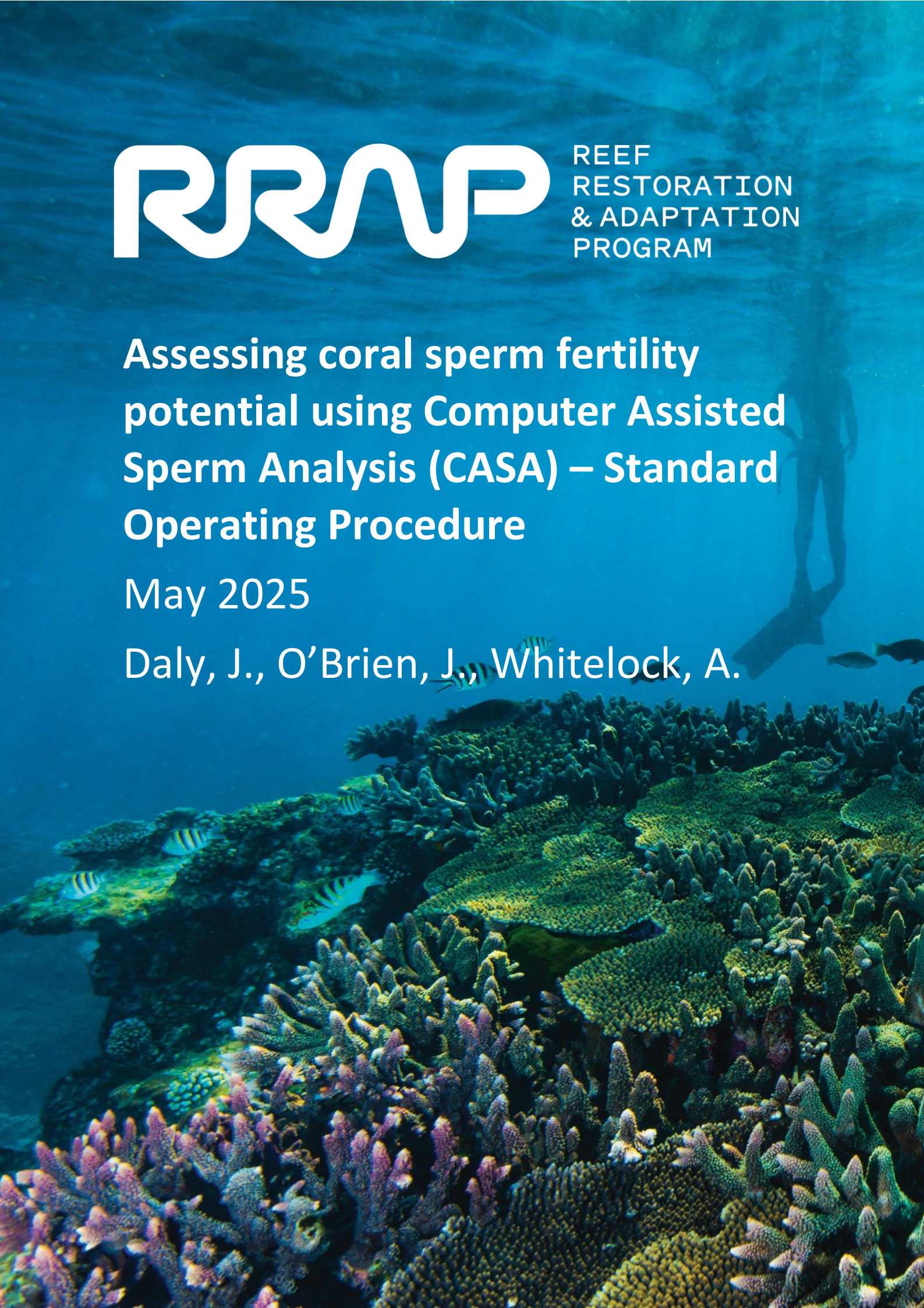
REEF RESTORATION & ADAPTATION PROGRAM

Assessing coral sperm fertility potential using Computer Assisted Sperm Analysis (CASA) – Standard Operating Procedure

May 2025

Daly, J., O'Brien, J., Whitelock, A.



Assessing coral sperm fertility potential using Computer Assisted Sperm Analysis (CASA) – Standard Operating Procedure

Enquiries should be addressed to: Jonathan Daly (jondaly@zoo.nsw.gov.au)

Cover Images: Coral Reef, Credit: Gary Cranitch, Queensland Museum

This report should be cited as

Daly, J., O'Brien, J., Whitelock, A. (May 2025) Reef Restoration and Adaptation Program – Assessing coral sperm fertility potential using Computer Assisted Sperm Analysis (CASA) – Standard Operating Procedure. (31 pp).

Acknowledgement

This work was undertaken for the Reef Restoration and Adaptation Program (RRAP). Funded by the partnership between the Australian Governments Reef Trust and the Great Barrier Reef Foundation, partners include: the Australian Institute of Marine Science, CSIRO, the Great Barrier Reef Foundation, Southern Cross University, the University of Queensland, Queensland University of Technology and James Cook University.

The RRAP partners acknowledge Aboriginal and Torres Strait Islander Peoples as the first marine scientists and carers of Country. We acknowledge the Traditional Owners of the places where RRAP works, both on land and in sea Country. We pay our respects to elders; past, present, and future; and their continuing culture, knowledge, beliefs, and spiritual connections to land and sea Country.

We specifically acknowledge and thank the following Traditional Owners of Country on which this report was prepared:

Location	**Traditional Owner Group**
Mosman, NSW	Cammeraygal
Kensington, NSW	Bidjigal

Table of Contents

List of Figures

List of Tables

1 Executive Summary

This Standard Operating Procedure outlines a validated and streamlined method for assessing coral sperm fertility potential using Computer Assisted Sperm Analysis (CASA). Accurate assessment of sperm sample quality and the inferred fertility potential is a critical component of coral cryopreservation workflows. Traditional manual techniques—such as haemocytometer-based cell counts and subjective motility estimates under phase microscopes—are time-consuming and can introduce variability. CASA offers an efficient, standardised approach that enables simultaneous and direct quantitation through video capture and software analysis of sperm concentration, motility, and movement parameters that provide information about the velocity, linearity and lateral displacement of sperm heads as they progress along their trajectories. Motion parameters of focus for coral sperm sample assessments include average path velocity (VAP), straightline velocity (VSL) and curvilinear velocity (VCL). Since its introduction to coral biobanking workflows in 2018, CASA has become a standard tool across programs in Australia, the USA, Mexico, and Curacao. Recent protocol refinements—including sperm activation with caffeine and the use of a Makler counting chamber—have further improved accuracy and reproducibility. The SOP is designed for cryopreservation practitioners and outlines a standardized approach for sperm analysis using CASA that is applicable to fresh and cryopreserved samples from both bundle spawning and gonochoric coral species.

2 Background

Assessment of sperm sample fertility potential (also referred to as 'sample quality' assessment) is an essential step in the cryopreservation pathway. Sperm sample quality is typically determined by estimating the number of cells in the sample (concentration), the proportion of cells that are actively moving (motility) and sperm motion parameters including velocity. For coral, these assessments have traditionally involved visualisation of a sample aliquot using a phase microscope to subjectively estimate the percentage of motile cells and vigour of movement, and manual cell counting using a haemocytometer to determine concentration. Computer assisted sperm analysis (CASA) provides an automated and standardised method to simultaneously estimate sperm concentration and motility and is much less time consuming than manual methods. The CASA system consists of a digital camera attached to a phase contrast microscope, and proprietary software (Hamilton-Thorne Inc., Beverly, MA, USA) that captures and analyses short videos of the sperm sample. The software also provides information on sperm kinematics including average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL), which can be used to further classify sperm as motile, progressively motile, slow, or static.

The use of CASA for coral sperm assessment was developed by scientists at the Smithsonian Institution and Taronga in 2018 (Zuchowicz et al. 2021), and is currently used as part of coral biobanking programs by groups in Australia, the United States of America, Mexico, and Curacao. Recent refinements to the protocol include the standardisation of sperm activation with caffeine for assessment, and the use of a Makler counting chamber to improve accuracy and reproducibility (Daly et al. 2024). The use of caffeine to activate motility removes the need to wait for sperm activation to occur naturally (which can take up to 30-60 min) and ensures that all samples are exhibiting maximal motility at the time of sample assessment. This approach means that samples can be prioritised for cryopreservation shortly after spawning, minimising potential detrimental impacts of sperm aging on subsequent fertility. Makler counting chambers are commonly used for sperm assessment in human and non-human animal reproduction, and provide an accurate and re-useable alternative to the single-use chamber slides commonly used for CASA. After workstation preparation, the process of analysing sperm samples using CASA begins with gamete bundle collection and sperm isolation, followed by sperm dilution and activation, and finally assessment of the sample and recording of sample quality data. The same process may be used to analyse the sperm motility of cryopreserved samples after thawing.

3 Objectives and Scope

The purpose of this SOP is to provide a standardised approach for the use of CASA to estimate sperm concentration, motility and movement data in coral colony samples. The SOP is aimed at cryopreservation practitioners who may use this SOP as part of a coral sperm biobanking workflow, but can be used by anyone requiring standardised methods for obtaining coral sperm quality information. The SOP provides a list of required equipment and steps for all stages of the sperm collection and analysis process, including thawing of cryopreserved samples for post-thaw analysis, along with additional information on best practices for consumables and equipment.

The SOP provides procedures for working with three sample types:

1. High concentration samples (~10^9 sperm/mL) collected from bundle spawning species (e.g., *Acropora spp.)*
2. Low concentration samples (≤10^7 sperm/mL) collected from gonochoric species (e.g., *Fungia fungites*) and species that do not package gametes in bundles (e.g., *Pocillopora meandrina*)
3. Samples that have been cryopreserved and thawed for assessment, primarily collected from bundle spawning species with high initial sperm concentrations.

4 Pre-requisites

4.1 Storage and use of fixed-depth chamber slides

The most commonly used slides for CASA are Leja 20-µm fixed-depth 4 chamber slides (SC-20-01-04-B; see **Annexure 1**), which are recommended by Hamilton-Thorne. These slides have a coating that is designed to promote consistent chamber filling across samples; however, this coating is susceptible to degradation caused by elevated temperature and humidity. To minimize this issue it is recommended to purchase slides in small batches to avoid long-term storage prior to use. Once opened, slide packs should be stored in an airtight container with desiccant packs. Chamber wick time should be checked on each day of use to ensure that slides are performing as expected, as an extended wick time indicates that degradation has occurred (**Annexure 2**). In some coral samples the slide coating can cause potentially motile sperm to stick to the slide (observable as sperm that are vibrating in place), impacting the accuracy of motility assessments. The addition of bovine serum albumin (BSA) to the activation solution helps to overcome this issue by preventing sperm from sticking to the slide to ensure consistency across samples. It is also recommended that slides are cleaned prior to use by wiping with a Kimwipe or other lint-free tissue to remove any residue from the manufacturing process.

4.2 Microscope and camera set-up

Hamilton-Thorne's Ceros II software is designed to work optimally with a 10× negative phase contrast microscope objective to give bright (white) sperm heads on a dark background. For accurate cell detection using CASA it is important that the microscope is set up for Kohler illumination and that the microscope phase rings are correctly aligned. Instructions for setting Kohler illumination are provided on the **Zeiss** and **Evident** (Olympus) microscope websites (Note: this step is not required for microscopes with a fixed condenser). Instructions for phase ring alignment and the specific phase ring centering telescope required are typically provided in the microscope user manual or are available from the manufacturer. Phase ring alignment and Kohler illumination should be checked and adjusted as required prior to sample collection.

The CASA system uses a monochrome digital microscope camera which, along with the green light source filter, provides a high-contrast image with clearly defined sperm heads to aid cell detection. The digital camera must be powered on prior to opening the Ceros II software in order to be detected by the software as an image source; however if powered on for prolonged periods, especially in warm environments, the camera may overheat. The camera should therefore only be powered on when required for sample assessment and powered off when not in use. Users may also choose to attach an adhesive pin grid heat sink to the front and/or back of the digital camera to help dissipate heat and reduce the risk of overheating.

4.3 Analysis settings

General instructions for using the Ceros II CASA system are provided in the **HT CASA Animal Motility user manual** provided by Hamilton-Thorne and should be consulted as a first step to setting up and using the CASA system. Specific settings for cell detection and motility in coral sperm samples are provided in **Annexure 3**, and further information on the development of these settings is provided in (Zuchowicz et al. 2021).

5 Identified Risks and Hazards

Table 1. Generalised hazard and risk table for handling and use of caffeine and BSA.

Task or Process	Hazards	Potential Consequence	Control Measures	Risk Category	Risk Rating		
					Consequence (of hazard)	Frequency (or likelihood)	Risk
Chemical use	Chemical spills, personal safety	Poisoning or incapacitation. Contamination of environment	• Store chemicals in original packaging in the appropriate environment as specified in the SDS • Use appropriate PPE as defined in the SDS (gloves, safety glasses, lab coat, enclosed shoes). • Label aliquots and prepared solutions with contents, date, and initials. • Refer to SDS and local laws for disposal.	Safety	Minor	Possible	Low

NOTE: Always consult the product SDS provided by the manufacturer prior to use and follow institutional guidelines for chemical use and disposal.

6 Equipment and Materials

6.1 Procedure equipment and consumables

Table 2. Equipment and consumables needed for assessment of coral sperm using CASA.

Item	Supplier	Product number
Solution preparation		
Vacuum pump*	Merck Millipore	WP6122050
Reusable bottle-top filtration system	Thermo Scientific	DS0320-5045
0.22-µm filter discs, mixed cellulose esters	Merck Millipore	GSWP04700
Filtered seawater	N/A	–
Caffeine	Sigma-Aldrich	C0750
BSA heat shock fraction	Sigma-Aldrich	A9647
Sample handling		
P2 0.2–2 µL pipettor	Gilson	F144054M
P10 1–10 µL pipettor	Gilson	F144055M
P20 2–20 µL pipettor	Gilson	F144056M
P200 20–200 µL pipettor	Gilson	F144058M
P1000 100–1000 µL pipettor	Gilson	F144059M
Eppendorf racks	Interpath	511029
Eppendorf 1.5-mL tubes	Eppendorf	30120086
15-mL tubes – racked	Thermo Scientific	339651
50-mL tubes – racked	Thermo Scientific	339653
Transfer pipettes	Thermo Scientific	Samco 202PK
100-µm filter baskets	Fisher Scientific	22363549
Sample thawing		
1.5 Litre Stainless Steel Thermos	Isosteel	VA-9683
Polystyrene container	Generic	–
Heated water bath	Genetics Australia	Dairymac Superthaw™
Cryovial rack	Simport	T315
Medium forceps	Generic	–
Thermometer	Generic	–
Sample assessment		
Phase contrast microscope	Zeiss	Axiolab 5
10× negative phase objective lens	Zeiss	441031-9910-710
Phase ring centering telescope	Generic	To fit Zeiss Axiolab 5
Monochrome digital microscope camera	Jai	CM-040GE
Leja 20-µm 4 chamber slides (SC-20-01-04-B)	IMV Technologies	025107
Makler sperm counting chamber (CASA)	IVF Store	SM-373
CASA system + laptop	Hamilton-Thorne	Ceros II
Spreadsheet software	Microsoft	Excel

NOTE: Supplier information and product numbers are for reference only and users may choose their own equivalent product and supplier.

*Small volumes of seawater can be hand-filtered through a 0.22-µm syringe filter using a rubber- and lubricant-free syringe.

6.2 Personal protective equipment (PPE) and other safety equipment

Table 3. PPE required for quality assessment of fresh or cryopreserved coral sperm using CASA.

Item	Manufacturer	Product number
Safety Glasses	Generic	–
Lab coat	Long sleeve, full length	–
Nitrile gloves	Generic	–
Cryogloves (pair)	Tempshield	Mid-arm

7 Steps for Implementation

7.1 Prepare Coral Sperm Activation Stock Solutions

NOTE: Disposable gloves should be worn when handling chemicals for preparation of the activation solution.

7.1.1 Prepare 30% BSA Stock Solution (5 mL)

1. Dissolve 1.5 g of BSA in 4 mL of sterile purified tissue culture water.

NOTE: Warming the solution (e.g., using a tube warmer set to 37 °C) with periodic gentle swirling will help to dissolve the BSA. Avoid agitating the solution (do not shake the vial) to minimize foaming.

2. Once in solution, make up to the final volume (5 mL) using tissue culture water.
3. Label the tube and store in the refrigerator (4 °C) for up to 2 weeks.

7.1.2 Prepare 60 mM Caffeine Stock Solution (25 mL)

1. Prepare 60 mM caffeine stock solution (25 mL) by dissolving 0.2913 g of caffeine in 24 mL of filtered seawater (FSW).
2. Once in solution, make up to the final volume (25 mL) using FSW.
3. Label the tube and store in the refrigerator for up to 1 month.

7.2 Prepare Sperm Activation Working Solution

NOTE: Sperm activation working solutions should be prepared fresh on each day of use. Store at room temperature (19–30 °C) and discard the unused solution at the end of the day.

7.2.1 For sperm samples at ~10^9 sperm/mL (e.g., bundle spawning species)

1. For 10 mL working solution, combine:
    - 2.0 mL of 60 mM caffeine stock solution
    - 0.3 mL of 30% BSA stock solution
    - 7.7 mL of FSW
2. Label tube with the date and "Sperm activation working solution – 0.9% BSA + 12 mM caffeine".

7.2.2 For sperm samples at ~10^7 sperm/mL (e.g., gonochoric species)

1. For a 10 mL working solution, combine:
    - 8.0 ml of 60 mM Caffeine Stock Solution
    - 0.265 ml of 30% BSA Stock solution
    - 1.735 ml FSW
2. Label tube with the date and “Sperm activation working solution – 0.9% BSA + 48mM caffeine”

7.3 Prepare CASA Software for Analysis

1. Ensure that the camera is powered on and connected to the computer.

2. Open the CASA software.
3. Open **Settings** and select the setup required for the target species, or select directly from the **Setups** dropdown menu on the menu bar at top left of the screen (e.g., *Acropora Zeiss 10x*).
4. In the **Analysis Info** section, input the required information for **Animal Species**, **Animal ID** (i.e., Colony ID), and **Study Number**.

NOTE: By default the metadata in the **Analysis info** section will be deleted after the captured videos are cleared. To retain some or all of this metadata across samples (e.g., for multiple samples from the same species), go to **Settings> Animal Motility> General> Info** and select the required fields in the **Persistent Info Fields** section.

5. Set the **Volume** as "1" to give sperm concentration as the number of cells/mL.
    - Alternatively, input the total sample volume to provide information on the total number of cells in the sample.
6. **OPTIONAL:** Input any pre-dilutions made to the sample prior to assessment in the **Sample:Extender** field. By default this value will be set to "0" (i.e., no sample pre-dilution).

NOTE: The number input into the **Sample:Extender** field acts as a multiplier to automatically calculate sperm concentration in the original sample prior to any dilutions made for assessment. Sperm concentration should be between 8–50 × 10^6/mL to ensure accurate cell detection and analysis by the CASA software. When samples are collected and prepared as described in **section 7.4**, this analysis concentration can usually be achieved by diluting an aliquot of the raw sperm sample with activation solution for assessment as described in **section 7.6**. In most cases, dilution with activation solution for assessment will be consistent across samples (e.g., 1:39 [sperm: activation solution, v/v] for sperm samples at ~10^9 sperm/mL); however, there are occasions when working with bundle-spawning species when a pre-dilution step is needed (i.e., prior to activation) to bring samples within the required concentration range for CASA. In this case the total dilution factor, including both the pre-dilution and the dilution with activation solution, must be calculated and input into the **Sample:Extender** field for each sample. An alternative approach is to use the default value of "0" in the **Sample:Extender** field for all samples regardless of the dilutions used and input the raw CASA data directly into a spreadsheet set up to calculate concentration in the sample (e.g., Daly et al. 2024).

7.4 Gamete Collection and Preparation

NOTE: Disposable gloves are not necessary for the collection and handling of gamete samples but may be worn if preferred. Hands should be washed thoroughly with fresh water, or gloves changed, between each gamete sample to avoid cross-contamination. Ambient temperature in the laboratory should be set to 24-28ºC when possible to avoid cold- or heat-shocking sperm.

7.4.1 For bundle-spawning species (e.g., *Acropora*)

1. Collect gamete bundles from the surface of the water using a clean, sanitized, plastic cup.
2. Using a 3-mL transfer pipette, transfer bundles into a labeled 50-mL tube at a ratio of 5 mL of gamete bundles over 5 mL of seawater (10 mL total volume).
3. Agitate the sperm-egg bundles by gently swirling the sample by hand until the bundles have broken apart.

7.4.2 For gonochoric species

1. Collect sperm from close to the polyp mouth using a 3-mL transfer pipette and transfer the sample into a labeled 50-mL tube.

7.4.3 Separate Sperm from Eggs

1. After bundle break up, let the sample sit for 2 minutes in a tube rack to allow eggs to float to the top of the sperm sample below.
    - Eggs will form a layer at the surface and individual eggs will be visible.
2. Gently aspirate the sperm sample from the bottom of the tube using a clean 3-mL transfer pipette.
3. Transfer the sperm sample to a 100-µm cell strainer sitting atop a new sterile 50-mL tube.
4. If necessary, gently tap the filter to encourage flow or use a clean transfer pipette to collect any residual sample from the underside of the filter.
5. Remove the filter, then cap the tube loosely to allow air exchange and label with the colony ID and relevant metadata from the original sample tube.

7.5 Cryopreserved samples

IMPORTANT: Protective eyewear must always be worn during sample thawing.

NOTE: Samples can be thawed in a commercially available heated water bath containing FSW (e.g., Dairymac Superthaw™) or by adding ambient FSW to an insulated container (e.g., a polystyrene box) and adjusting the temperature with boiling tap water from a kettle or similar. Filtered seawater is used in the water bath to minimize the impact on sperm quality if water enters the sample vial during thawing.

1. Remove cryopreserved samples from storage and transfer to a small insulated container containing with liquid nitrogen (e.g., a polystyrene box).

NOTE: Carefully inspect the cryovials to ensure that they do not contain liquid nitrogen. Cryovials that do not have liquid nitrogen inside will float at the surface of the liquid nitrogen, whereas cryovials that sink have been improperly sealed allowing liquid nitrogen to enter. To remove liquid nitrogen from cryovials, hold the sample in the vapour phase (~–150^{o}C) using forceps and carefully loosen the cap, then decant the liquid nitrogen inside and re-cap the cryovial. Thawing cryovials that contain liquid nitrogen may result in explosion and injury.

2. Add FSW to the water bath and place a thermocouple probe in the water to monitor temperature.
3. Depending on the type of water bath used, set the heated water bath temperature to 30ºC or adjust the water temperature to 30ºC using hot water.
4. Using medium or long forceps, remove the sample vial and flick quickly to remove any liquid nitrogen on the surface, then plunge the sample into the water bath and start a count-up timer
5. Gently swirl the sample in the water to promote even warming, periodically checking that the cryovial lid is firmly tightened as it may become loose during the thawing process.
6. After approximately one minute gently invert the vial 180 degrees to check whether the sample has started to melt (the sample will begin to slide inside the cryovial).
7. Continue swirling the sample in the water bath and repeat this inversion approximately every 10s to promote even warming until the sample is completely thawed.
8. Thawing will take around two minutes in total and the final sample temperature should be around 25–30ºC (the sample should feel slightly cool but not cold in the hand).

7.6 Prepare Sperm Sample for CASA

NOTE: For batch processing, pre-load 1.8-mL microcentrifuge tubes with the required amount of activation solution then add sperm directly to the activation solution and mix as described.

7.6.1 For sperm samples at ~10^9 sperm/mL (e.g., bundle spawning species)

1. Mix the sperm sample well and transfer a 10 µL aliquot to an empty 1.8-mL microcentrifuge tube.
2. Immediately add 0.390 mL of sperm activation working solution (from **step 7.2.1**) dropwise over 5–10 seconds, gently mixing sperm into the solution.
    - This gives a dilution ratio of 1:39 (sperm: activation solution, v/v) and provides an appropriate concentration for CASA assessment, assuming a raw sperm concentration of approximately 2 × 10^9 sperm/mL. To automatically calculate raw sperm concentration, input "40" in the **Sample:Extender** field (**see Section 7.3**).
3. Invert the tube five times and flick the tube lid prior to opening to settle the solution into the bottom of the tube.

NOTE: If during analysis the concentration exceeds the required range (8–50 × 10^6/mL) the software will display an error message. If this occurs, dilute an aliquot of the raw sperm sample with FSW (start with a 1:1 dilution to halve the concentration), then reanalyse the diluted sample using a 1:39 dilution with activation solution as described, ensuring that the new dilution factor is updated in the **Sample:Extender** field and/or data spreadsheet (see **section 7.3**).

7.6.2 For sperm samples at ~10^7 sperm/mL (e.g., gonochoric species)

1. Mix the sperm sample well and transfer a 90 µL aliquot to an empty 1.8-mL microcentrifuge tube.
2. Immediately add 10 µL of sperm activation working solution (from **step 7.2.2**) and gently flick the tube five times to mix.
    - This gives a dilution ratio of 9:1 (sperm: activation solution, v/v) and provides an appropriate concentration for CASA assessment, assuming a raw sperm concentration is ~5 x 10^7 sperm/ml or less. To automatically calculate raw sperm concentration, input "0.11" in the **Sample:Extender** field (**see Section 7.3**).

7.6.3 For cryopreserved samples

1. Immediately after thawing, mix the sperm sample well and transfer a 10 µL aliquot to an empty 1.8-mL microcentrifuge tube.
2. Add 90 µL of sperm activation working solution (from **step 7.2.1**) and gently flick the tube five times to mix.
    - This gives a dilution ratio of 1:9 (sperm: activation solution, v/v) and provides an appropriate concentration for CASA assessment, assuming a post-thaw sperm concentration of approximately 0.5 x 10^9 sperm/mL. To automatically calculate sperm concentration in the cryovial, input "10" in the **Sample:Extender** field (**see Section 7.3**).
    - In some cases, post-thaw sperm may show sensitivity to dilution due to osmotic factors related to the cryopreservation process. If sensitivity to dilution is suspected or observed, activation solution should be added slowly, dropwise over 30s-1 min, and the extended dilution time should be recorded in datasheet or spreadsheet associated with the sample.

7.7 CASA Assessment

7.7.1 If using 20-µm fixed-depth slides (e.g., Leja):

1. Open Settings at the top right-hand corner of the screen then select the **Setup** for the target species under **Settings> Animal Motility> Setups** and scroll down to the **Chamber** section.
2. Set **Chamber Type** to "Capillary" and **Chamber Depth** to "20 µm".

3. Load the slide with 4 µL of activated sperm solution (**Figure 1**).
4. Allow the chamber to fill completely, then carefully blot away excess sample.

7.7.2 If using a Makler counting chamber:

1. Open **Settings** at the top right-hand corner of the screen then select the **Setup** for the target species under **Settings> Animal Motility> Setups** and scroll down to the **Chamber** section.
2. Set **Chamber Type** to “Droplet” and **Chamber Depth** to “10 µm”.
3. Place 3 µL of activated sperm suspension on the chamber (**Figure 1**).
4. Carefully position the cover slip so that the glass is in even contact with the contact posts on the chamber.

NOTE: When viewed at the correct angle, Newton’s Rings should be visible as a series of concentric rings on the contact posts, indicating that the coverslip and chamber are correctly aligned.

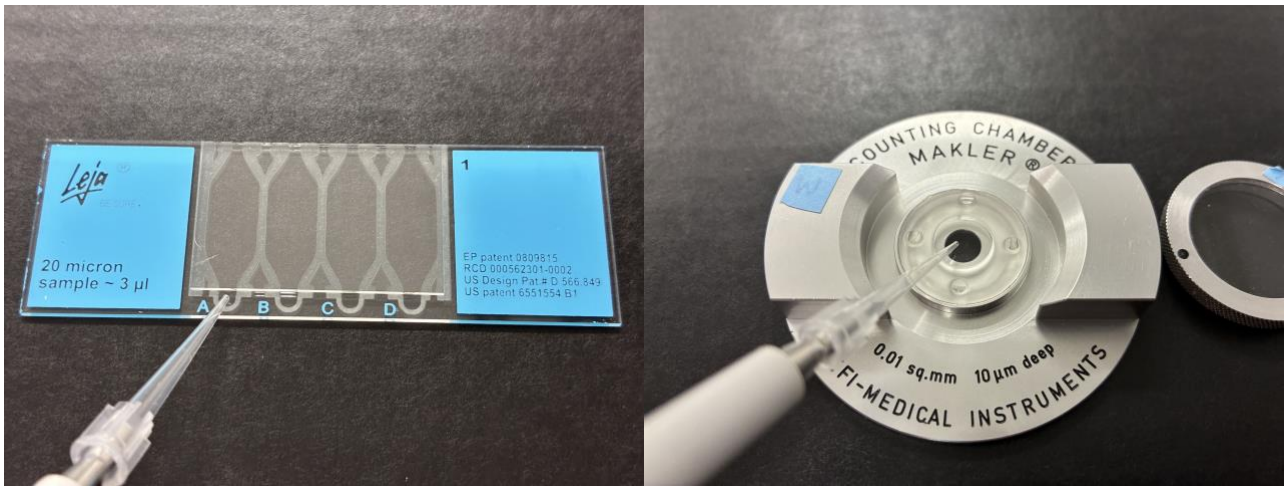


*Figure 1: Sample loading into a 20-µm fixed-depth Leja slide (**left**) and a Makler counting chamber (**right**).*

7.7.3 CASA Data acquisition

1. Position the sample chamber under the microscope objective lens.
2. Adjust the microscope light intensity so that the illumination value on the right hand side of the Motility Toolbar is “Acceptable” (**Figure 2**).

NOTE: Ensuring that illumination levels are in the “Acceptable” range helps to maintain consistent cell detection across samples. The “Acceptable” range is defined by setting the **Max Photometer** and **Min Photometer** values in the **Illumination** section of the Analysis Setup and may need to be adjusted to ensure that they correspond to an appropriate level of illumination for the microscope system used.

3. Focus the image on screen.
4. Ensure the sample region is at least two microscope fields away from the edges and ends of the slide chamber.
5. Select **Live Overlay** in the Motility Sub-menu on the left-hand side of the screen in the HTCASA II software and adjust the microscope focus to ensure sperm heads show blue (**Figure 2**).
6. Click the **Capture** button (above **Live Overlay**) in the Motility Sub-menu on the left-hand side of the screen in the HTCASA II software to analyze the current field (**Figure 2**).

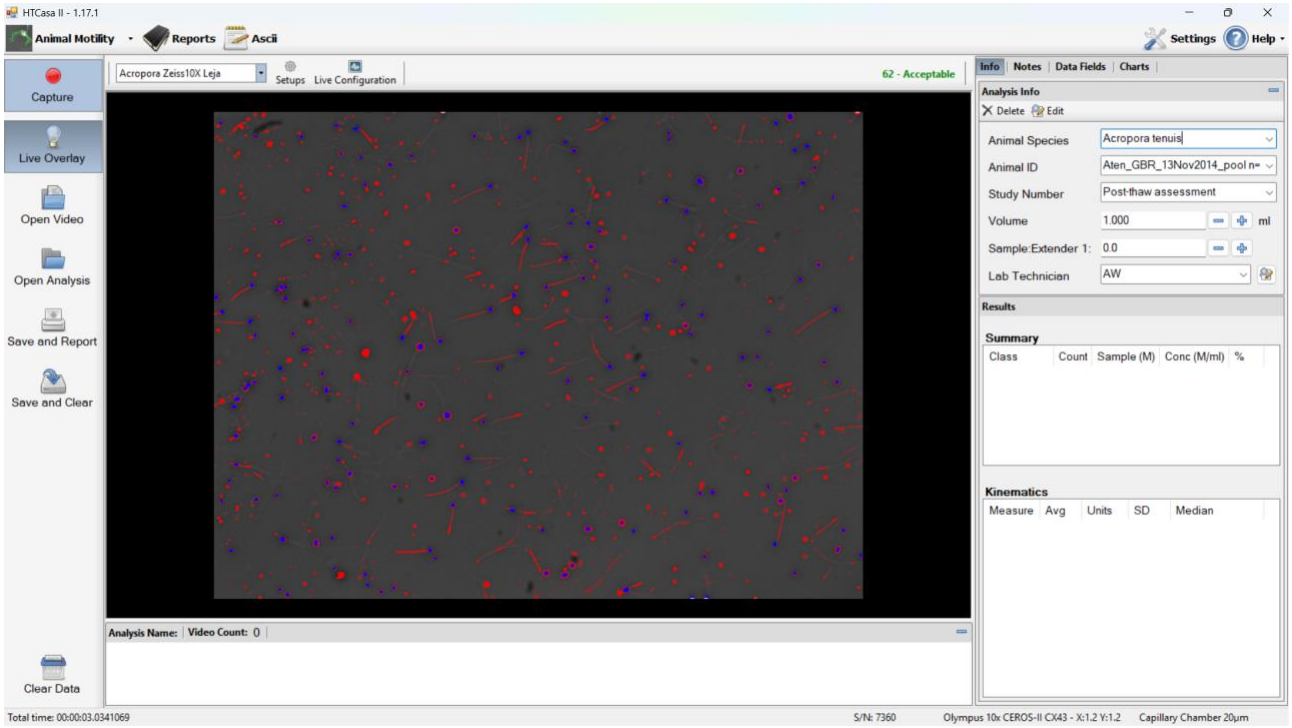


Figure 2. HT CASA II analysis screen showing the Live Overlay view of a post-thaw coral sperm sample.

7. Move the microscope stage to a new field and repeat until at least 5 fields of view and 200 cells have been captured.

NOTE: Use a consistent pattern of fields for all samples (e.g., five across the width of the sample chamber, or three across and two above/below).

8. Record the values for **Total Conc (M/ml)** (total concentration), **Motile (%)** (total motility), and **Progressive (%)** (progressive motility) shown in the **Summary Results** panel (**Figure 3**) into a datasheet or spreadsheet (e.g., Daly et al. 2024).

NOTE: If the **Sample:Extender** field is set to the default value of "0" (see **section 7.3**) then sperm concentration will need to be calculated by multiplying the **Total Conc (M/ml)** value by the dilution factor used for sperm activation. Be sure to include any additional dilution factors used to adjust sperm concentration in this calculation. Additional CASA metrics such as average path velocity (VAP), straightline velocity (VSL) and curvilinear velocity (VCL) (Holt et al. 2007) can be recorded at this stage or may be retrieved from saved CASA files at a later time.

4. Write the sperm concentration on the filtered sperm sample tube.

5. Confirm that the **Animal Species**, **Animal ID** (i.e., Colony ID), and **Study Number** information in the **Analysis Info** section are correct.

6. Select **Save and Clear** at the bottom of the Motility Sub-menu on the left-hand side of the screen in the HT CASA II software.

7. If using a Makler counting chamber, clean the chamber and coverslip with 70% ethanol or isopropyl alcohol, then distilled water, and wipe with a lint-free tissue.

8. Repeat the procedure for the remaining sperm samples.

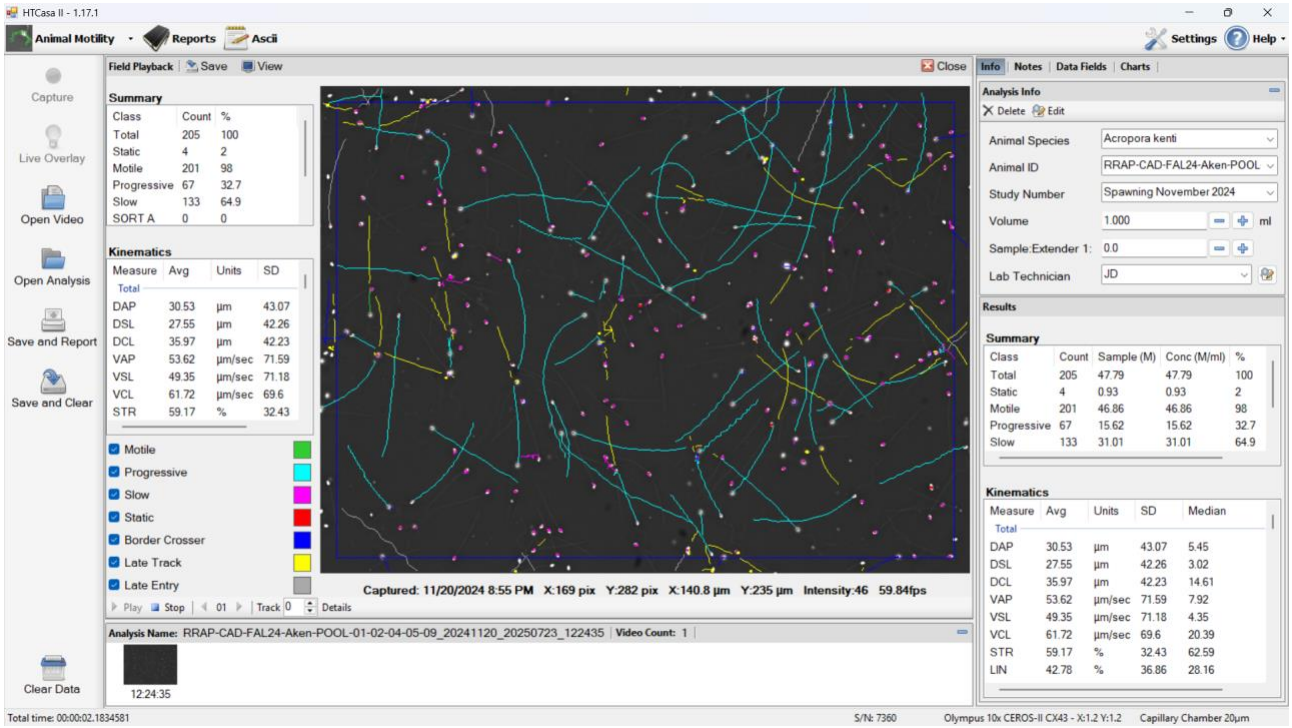


Figure 3: HT CASA II field playback screen showing classification and tracking of all sperm detected in the image area (centre) along with sample quality information in the Summary Results and Kinematics sections (right).

8 References

Daly J, Hobbs R, Zuchowicz N, Hagedorn M, O'Brien JK. 2024. A Semi-Automated Workflow for the Cryopreservation of Coral Sperm to Support Biobanking and Aquaculture. *Journal of Visualized Experiments*, 208.

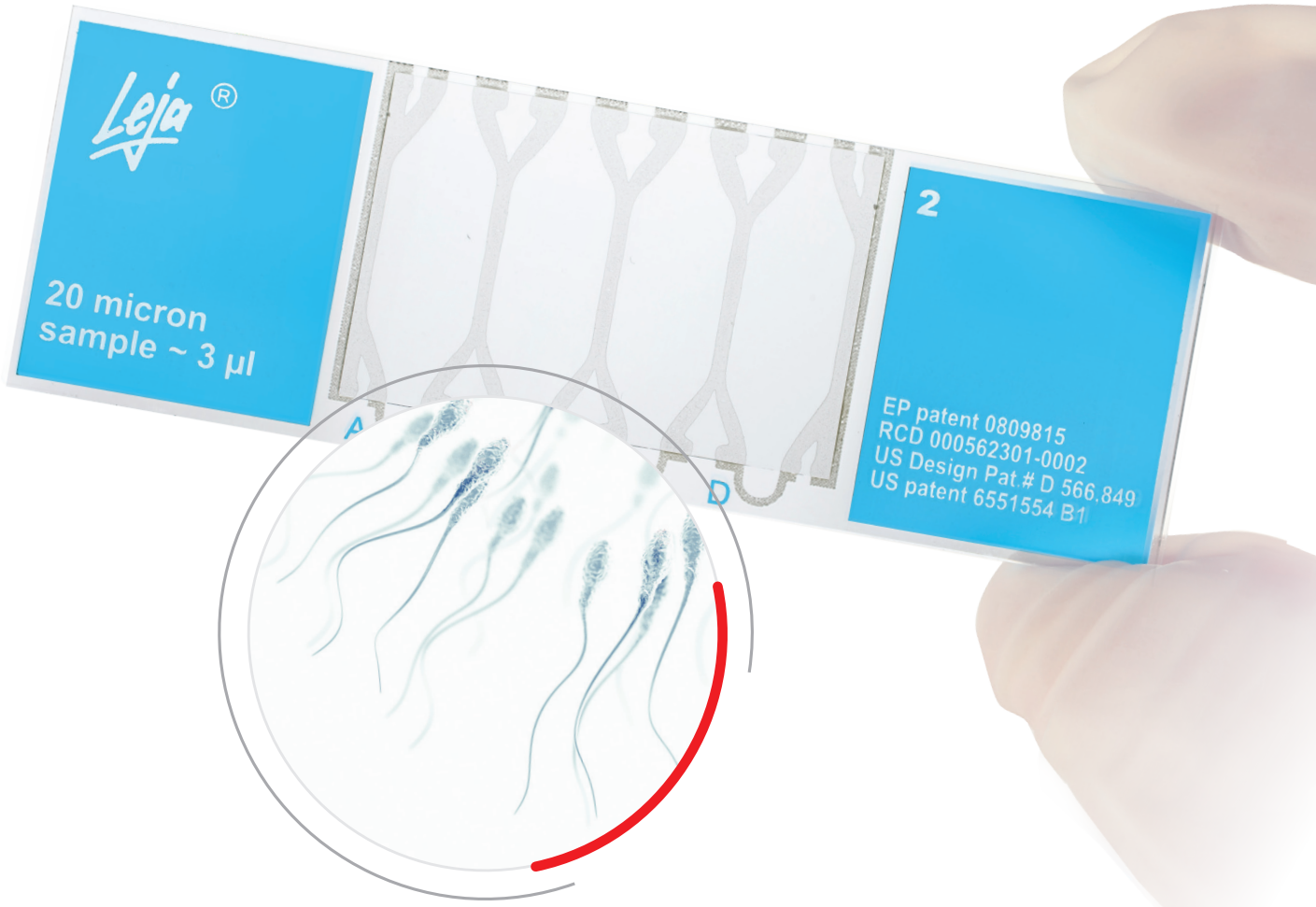
Holt WV, O'Brien JK Abaigar T. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. *Reproduction, fertility and development*, *19*(6): 709-718.

Zuchowicz N, Daly J, Bouwmeester J, Lager C, Henley EM, Nunez Lendo CI, Hagedorn M. 2021. Assessing coral sperm motility. *Scientific Reports* 11: 61.

Annexure 1

Leja®

The most accurate fixed-depth slide on the market



imv TECHNOLOGIES

Leja slide: the must-have slide for CASA

To get the most from CASA systems (such as IVOS II™ and CEROS II™), it is vital to use calibrated slides.
Only calibrated slides provide the accuracy and precision needed to reliably analyze semen.

But not all calibrated slides are of the same quality.

The Leja slide is more accurate and more reliable than its competitors. And, it is a perfect tool to use with most CASA systems. It allows for the standardization of analyses for repeatable, objective measurements.

This is why it is widely used by semen production centers, veterinarians, and research centers.

High technology and accuracy

Semen analysis accuracy is crucial because it has a direct impact on the results calculated by CASA systems. This, in turn, may affect economic returns.

Constant chamber depth

A calibrated slide's accuracy is related to the consistency of chamber depth.
Leja has developed a production method that ensures constant depth. Leja is the only manufacturer capable of delivering certification to prove it.

To achieve this level of quality, Leja has established strict controls:

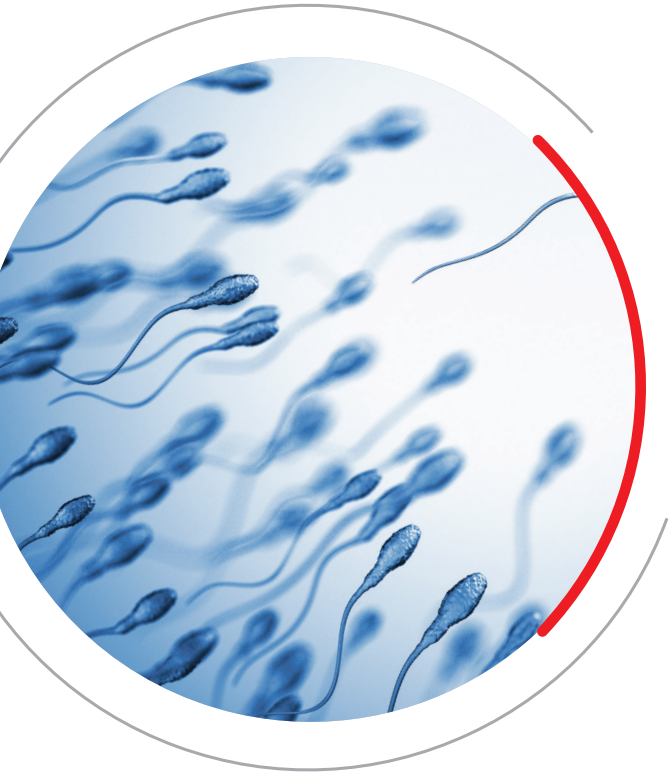
- inspection and tracking of raw materials
- toxicity checks
- depth checks
- 18-point visual inspection

High-tech materials

The high quality of the glass being used ensures a faithful image of sperm cells, a quality required for CASA analyses.

Producing slides in a clean room minimizes the risk of dust being deposited on the slide, thereby preventing reading errors and making the analyses even more accurate.

All elements used are non-spermicidal, and a semen toxicity check is performed on every batch of slides produced.
A certificate of analysis is issued for each batch.



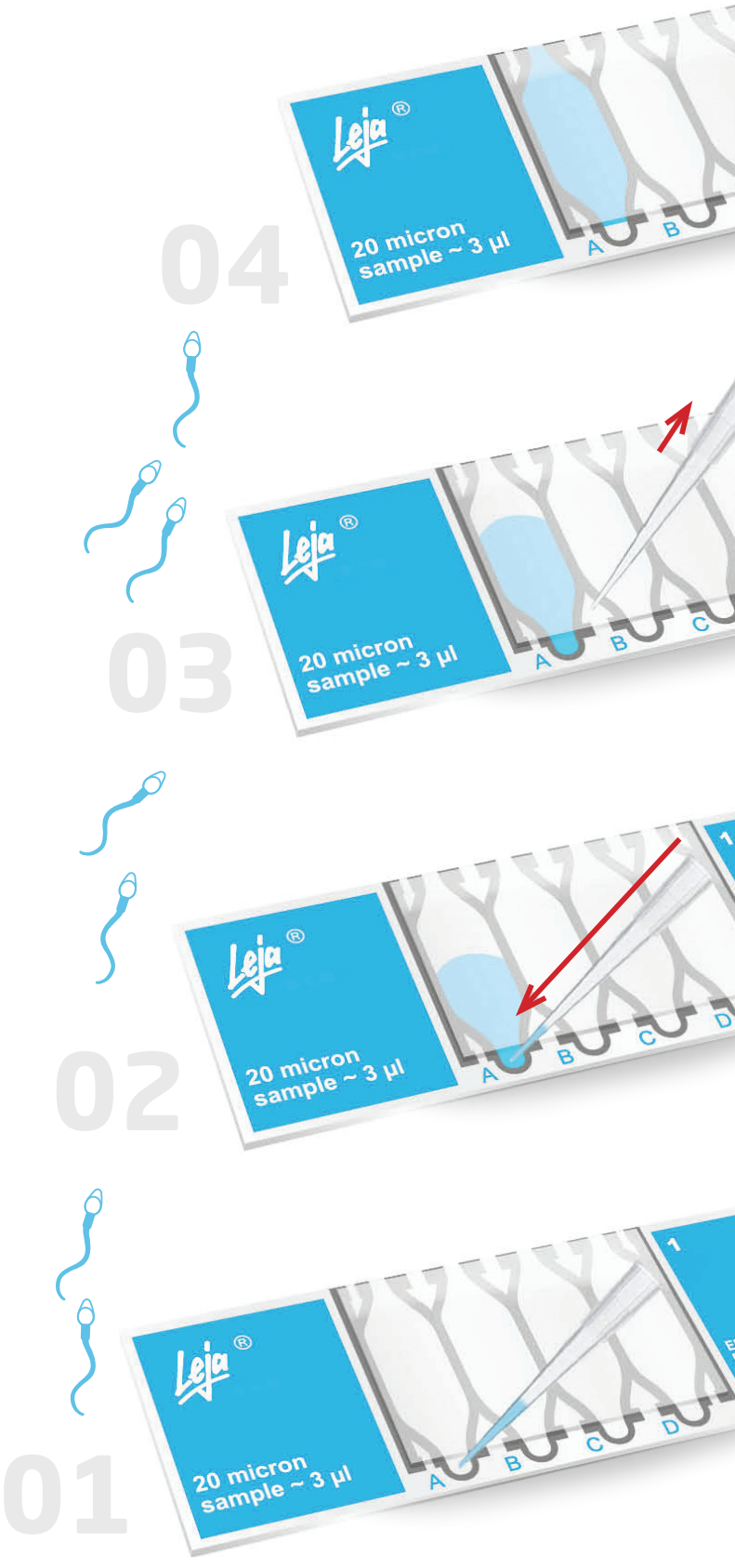
Controlled filling of the Leja slide chamber

Filling the chamber is one of the most important steps to ensure for reliable concentration and sperm mobility measurements.

To guarantee repeatability, the filling of the chambers with capillary action is made possible by our proprietary glass coating materials.

Spermatozoa do not stick to the slide, and are evenly distributed. Bubble formation is minimized with our technology.

These features allow standardized procedures and accurate measurements and may result in higher returns on investment for the user.



IMV, always at your side

Gain our expertise

IMV Technologies has solid expertise in numerous species and has developed leading technologies for various stages of reproduction.

IMV wants to share this expertise with users and help them through training and technical assistance.

Train your teams

A tool's performance is nothing without the skills of those who use it.
This is why IMV offers training courses to help technicians improve their knowledge and skills in the handling of slides. Our courses also focus on the standardization of procedures for CASA systems.

- Leja 4 chamber slides, 20 µm - 025107
- IVOS II™ - 024911
- IVOS II™ Fluorescence - 024910
- CEROS II™ Zeiss - 024905
- IVOS II™ and CEROS II™ installation and training - 026349

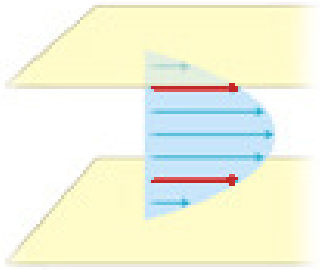
Annexure 2

INSTRUCTIONS for Segre Silberberg correction factor

Leja® disposable counting chambers

What is the Segre Silberberg effect?

Capillary flow into a 20 µm counting chamber follows a classical Poiseuille flow. The flow of the fluid is dominated by its viscosity. A maximum velocity is reached at exactly half the depth of the chamber (see diagram) while the velocity at the chamber walls equals 0 µm sec-1.



One can image that the sperm-cells in the middle of the chamber-height move faster than the ones near the wall. It has been shown that all sperm-cells move to two planes at equidistance from each chamber wall (depicted by the red arrows in the diagram).
The distance of these planes from the wall (β) is depending on a few parameters:

- development of full Poiseuille flow
- chamber height
- surface properties of the counting chamber
- surface tension
- fluid viscosity
- flow velocity
- diameter of sperm-cell head

How to correct for the Segre Silberberg effect?

Because the sperm-cells in the two Segre Silberberg planes move faster than the average fluid velocity, there is an accumulation of sperm-cells at the filling front. When measuring the sperm concentration in the center of the Leja® slide, an underestimation of the concentration takes place.
Luckily, this is a constant underestimation that can be corrected for.

Since all variables are kept constant the only variable that affects the dimension of the Segre Silberberg effect is the viscosity of the sample. Filling time and viscosity are closely associated. By measuring the filling time of the Leja chamber with a capillary length of 21 mm, the Segre Silberberg compensation factor *Sx* for that specific sample can be read from the conversion table.

.

The correction factor is only a constant when using a linear flow slide like the Leja® new design 2 chamber and the Leja® 4 chamber slides. Working with these slides will yield in an exact count that matches hemocytometer counts.
Due to the chamber height of 100 microns, the Segre Silberberg effect is negligible.

How to measure the filling time?

Load the sample into the chamber using a positive displacement pipette. Hold the pipette at an approximate angle of 45° and slowly deposit 1.5 times the indicated volume in the entry port.
Start measuring as soon as the capillary filling starts and stop when the liquid has filled the counting chamber completely.

Correction Factor conversion table

Filling time (sec.)	*Sx*	*Viscosity 2 chamber slide (cP)*	*Viscosity 4 chamber slide (cP)*
2.0	*1.32*		
2.1	*1.31*		
2.2	*1.30*		
2.3	*1.29*		
2.4	*1.28*		
2.5	*1.27*		
2.6	*1.26*		
2.8	*1.25*		
2.9	*1.24*		
3.2	*1.23*	*2.16*	*2.25*
3.4	*1.22*	*2.21*	*2.32*
3.6	*1.21*	*2.27*	*3.38*
3.8	*1.20*	*2.32*	*2.44*
4.0	*1.19*	*2.37*	*2.50*
4.2	*1.18*	*2.43*	*2.56*
4.5	*1.17*	*2.51*	*2.66*
5.0	*1.16*	*2.64*	*2.81*
5.3	*1.15*	*2.72*	*2.90*
5.5	*1.14*	*2.77*	*2.96*
6.0	*1.13*	*2.90*	*3.12*
7.0	*1.11*	*3.16*	*3.43*
8.0	*1.10*	*3.43*	*3.74*

These values only hold for the Leja 20 micron chambers (SC-20-01-02-B and SC-20-01-04-B)

Annexure 3

CASA settings for analysis of coral sperm fertility potential using Leja 20-µm fixed-depth 4 chamber slides

(Setup)	
(Name)	**Acropora Zeiss10X Leja**
Analysis Limits	
Min Motility Percent	**0**
Min Progressive Percent	**0**
Min Total Count	**200**
Calibration	
Objective	**0: Zeiss 10x NH CEROS-II**
Objective Magnification X	1.2
Objective Magnification Y	1.2
Camera	
Exposure (Ms)	**8**
Gain	**100**
Integrate Enabled	**False**
Integrate Time (Ms)	**500**
Cell Detection	
Elongation Max (%)	**100**
Elongation Min (%)	**50**
Enable Advanced Tail Det	**False**
Head Brightness Min	**50**
Head Size Max (µm²)	**20**
Head Size Min (µm²)	**4**
Large Object Min Area Mic	**1000**
Large Object Removal Ena	**False**
Min Cell Brightness Auto M	**Manual**
Static Tail Filter	**False**
Tail Brightness Min	**80**
Tail Min Brightness Auto Of	**8**
Tail Min Brightness Mode	**Auto - First Frame**
Chamber	
Capillary Correction	**1.3**
Chamber Depth (µm)	**20**
Chamber Type	**Capillary**
Illumination	
Histogram Smooth Width	**0**
Max Photometer	**65**
Min Photometer	**50**

Kinematics	
Cell Travel Max (µm)	**10**
Enable Motile Static Collisic	**False**
Motile cells require a tail	**False**
Motile Require Tails Max V	**0**
Progressive STR (%)	**0**
Progressive VAP (µm/s)	**80**
Slow VAP (µm/s)	**20**
Slow VSL (µm/s)	**30**
Static Algorithm	**Width_Multiplier**
Static VAP (µm/s)	**0**
Static VSL (µm/s)	**0**
Static Width Multiplier	**0.8**
Morph	
Display Morph Results	**True**
DMR Confidence (%)	**1**
DMR Droplet to tail end Ma	**1**
DMR Tail Length Max (µm)	**0**
DMR To Static	**False**
Droplet Confidence (%)	**1**
Droplet Distal Distance Mir	**0**
Droplet Proximal Head Len	**0**
Min Tail Length (µm)	**0**
Morph Normal Minimum Pe	**0**
Tail Bend Angle Averaging	**0**
Tail Bending Angle Rate M	**1**
Tail Bent Confidence (%)	**1**
Tail Coiled Angle Min (°)	**1**
Tail Coiled Confidence (%)	**1**
Tail Confidence (%)	**1**
ViadentCategory	
Viadent Fluorescing Sperm	**NonViable**
Video Capture	
Frame Capture Speed (Hz)	**60**
Frame Count	**45**
Imaging Type	**NegativePhase**

REEF RESTORATION & ADAPTATION PROGRAM

Australian Government

Great Barrier Reef Foundation

Australian Government

Australian Institute of Marine Science

CSIRO

James Cook University Australia

QUT

Southern Cross University

The University of Queensland Australia