

Journal of Visualized Experiments

Medium-throughput screening assays for assessment of effects on Ca²⁺-signaling and acrosome reaction in human sperm

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59212R2
Full Title:	Medium-throughput screening assays for assessment of effects on Ca ²⁺ -signaling and acrosome reaction in human sperm
Keywords:	Sperm CatSper Progesterone Screening Assay Calcium Acrosome
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Copenhagen. Denmark

TITLE:

Medium-Throughput Screening Assays for Assessment of Effects on Ca^{2+} -Signaling and Acrosome Reaction in Human Sperm

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KEYWORDS:

Sperm, Semen, CatSper, Calcium, Acrosome, Fertility

SUMMARY:

Here, two medium-throughput assays for assessment of effects on Ca^{2+} -signaling and acrosome reaction in human sperm are described. These assays can be used to quickly and easily screen large amounts of compounds for effects on Ca^{2+} -signaling and acrosome reaction in human sperm.

ABSTRACT:

Ca^{2+} -signaling is essential to normal sperm cell function and male fertility. Similarly, the acrosome reaction is vital for the ability of a human sperm cell to penetrate the zona pellucida and fertilize the egg. It is therefore of great interest to test compounds (e.g., environmental chemicals or drug candidates) for their effect on Ca^{2+} -signaling and acrosome reaction in human sperm either to examine the potential adverse effects on human sperm cell function or to investigate a possible role as a contraceptive. Here, two medium-throughput assays are described: 1) a fluorescence-based assay for assessment of effects on Ca^{2+} -signaling in human sperm, and 2) an image cytometric assay for assessment acrosome reaction in human sperm. These assays can be used to screen a large number of compounds for effects on Ca^{2+} -signaling and acrosome reaction in human sperm. Furthermore, the assays can be used to generate highly specific dose-response

curves of individual compounds, determine potential additivity/synergism for two or more compounds, and to study the pharmacological mode of action through competitive inhibition experiments with CatSper inhibitors.

INTRODUCTION:

The purpose of the two assays described here is to examine effects on Ca^{2+} -signaling and acrosome reaction in human sperm, as has been shown for multiple compounds in several publications employing these assays¹⁻⁷. Ca^{2+} -signaling and the acrosome reaction are both vital to normal human sperm cell function and male fertility.

The overall goal of a human sperm cell is to fertilize the egg. To be able to successfully and naturally fertilize the egg, the functions of the sperm cell must be regulated tightly during the journey of the sperm cell through the female reproductive tract^{8,9}. Many of the sperm cell functions are regulated via the intracellular Ca^{2+} -concentration $[\text{Ca}^{2+}]_i$ (e.g., sperm motility, chemotaxis, and acrosome reaction)¹⁰. Also, a maturation process called capacitation, which renders the sperm cell capable of fertilizing the egg, is partly regulated by $[\text{Ca}^{2+}]_i$ ¹⁰. Ca^{2+} -extruding Ca^{2+} -ATPase pumps¹¹ maintain an approximately 20,000 fold Ca^{2+} -gradient over the human sperm cell membrane, with a resting $[\text{Ca}^{2+}]_i$ of 50-100 nM. If Ca^{2+} is allowed to cross the cell membrane (e.g., through the opening of Ca^{2+} -channels), a sizeable influx of Ca^{2+} occurs, giving rise to an elevation of $[\text{Ca}^{2+}]_i$. However, the sperm cell also carries intracellular Ca^{2+} -stores, which can release Ca^{2+} and, therefore, also give rise to an elevation of $[\text{Ca}^{2+}]_i$ ¹². Interestingly, all channel-mediated Ca^{2+} -influx in human sperm cells has so far been found to occur via CatSper (**C**ationic channel of **S**perm), which is only expressed in sperm cells¹¹. In human sperm cells, CatSper is activated by the endogenous ligands progesterone and prostaglandins through distinct ligand binding sites¹³⁻¹⁵, leading to a rapid Ca^{2+} -influx into the sperm cell. Two main sources near the egg provide high levels of these endogenous ligands. One is the follicular fluid that contains high levels of progesterone¹⁶. The follicular fluid is released from mature follicles together with the egg at ovulation and mixes with the fluid within the oviducts¹⁷. The other main source is the cumulus cells that surround the egg and release high levels of progesterone and prostaglandins. The progesterone-induced Ca^{2+} -influx in the sperm cells has been shown to mediate chemotaxis towards the egg^{9,18}, control sperm motility^{19,20} and stimulate the acrosome reaction²¹. Triggering of these individual $[\text{Ca}^{2+}]_i$ -regulated sperm functions in the correct order and at the correct time is crucial for fertilization of the egg⁸. In line with this, a suboptimal progesterone-induced Ca^{2+} -influx has been found to be associated with reduced male fertility²²⁻²⁹ and functional CatSper is essential for male fertility^{26,30-36}.

As the sperm cells reach the egg, a sequence of events must take place for fertilization to occur: 1) The sperm cells must penetrate the surrounding cumulus cell layer, 2) bind to the zona pellucida, 3) exocytose the acrosomal content, the so-called acrosome reaction³⁷, 4) penetrate the zona pellucida, and 5) fuse with the egg membrane to complete fertilization³⁸. To be able to go through these steps and fertilize the egg, the sperm cell must first undergo capacitation¹¹, which begins as the sperm cells leave the seminal fluid containing "decapacitating" factors³⁹ and swim into the fluids of the female reproductive tract with high levels of bicarbonate and albumin³⁷. Capacitation renders the sperm cells able to undergo hyperactivation, a form of

motility with a vigorous beating of the flagellum, and acrosome reaction³⁷. Hyperactivated motility is required for penetration of the zona pellucida⁴⁰, and the acrosome contains various hydrolytic enzymes that aid this penetration process⁴¹. Additionally, the acrosome reaction renders the sperm cells capable of fusing with the egg by exposing specific membrane proteins on the sperm surface necessary for sperm-egg fusion⁴². Consequently, the ability to undergo hyperactivation and acrosome reaction are both required for successful fertilization of the egg^{40,42}. Contrary to what has been seen for mouse sperm cells^{43–45}, only human sperm cells that are acrosome-intact can bind to the zona pellucida⁴⁶. When the human sperm cells are bound to the zona pellucida they have to undergo the acrosome reaction both to penetrate the zona pellucida⁴¹ and to expose specific membrane proteins that are needed for the fusion with the egg³⁸. The timing of the acrosome reaction in human sperm is thus critical for fertilization to occur.

As described above, Ca^{2+} -signaling is vital for normal sperm cell function⁸, and it is, therefore, of great interest to be able to screen large numbers of compounds for effects on Ca^{2+} -signaling in human sperm cells. Similarly, as only human sperm cells that undergo acrosome reaction at the right time and place can penetrate the zona pellucida and fertilize the egg^{46,47}, it is also of great interest to be able to test compounds for their ability to affect the acrosome reaction in human sperm. To this end, two medium-throughput screening assays are described: 1) an assay for effects on Ca^{2+} -signaling in human sperm cells, and 2) an assay for the ability to induce acrosome reaction in human sperm cells.

Assay 1 is a medium-throughput Ca^{2+} -signaling assay. This fluorescence plate reader-based technique monitors changes in fluorescence as a function of time simultaneously in multiple wells. The Ca^{2+} -sensitive fluorescent dye, Fluo-4 has a K_d for $\text{Ca}^{2+} \approx 335$ nM and is cell-permeant in the AM (acetoxymethyl) ester form. Using Fluo-4, it is possible to measure changes in $[\text{Ca}^{2+}]_i$ over time and after the addition of compounds of interest to the sperm cells. The assay was developed by the lab of Timo Strücker in 2011¹³ and has since been used in several studies to screen compounds for effects on Ca^{2+} -signaling in human sperm^{1–5}. Also a similar method has been used to screen multiple drug candidates⁴⁸. In addition, this assay is also useful for assessing the pharmacological mode of action^{1–5}, dose-response curves^{1–5}, competitive inhibition^{1,2}, additivity^{1,2}, and synergism³ of compounds of interest.

Assay 2 is a medium-throughput acrosome reaction assay. This image cytometer-based technique measures the amount of viable acrosome reacted sperm cells in a sample, using three fluorescent dyes: propidium iodide (PI), FITC-coupled lectin of *Pisum sativum* (FITC-PSA), and Hoechst-33342. The assay was modified from a similar flow cytometry-based method by Zoppino et al.⁴⁹ and has been used in several studies^{6,7}. As for the Ca^{2+} -signaling assay, this acrosome reaction assay could also be used to assess dose-response curves, inhibition, additivity, and synergism of compounds of interest.

PROTOCOL:

The collection and analysis of human semen samples in the protocols follows the guidelines of

the Research Ethics Committee of the Capital Region of Denmark. All semen samples have been obtained after informed consent from volunteer donors. After delivery, the samples were fully anonymized. For their inconvenience each donor received a fee of 500 DKK (about \$75 US dollars) per sample. The samples were analyzed on the day of delivery and then destroyed immediately after the laboratory experiments.

NOTE: The medium throughput Ca^{2+} -signaling assay is described in steps 4-5 and the medium throughput acrosome reaction assay in steps 6-7. The protocol to prepare human tubal fluid (HTF⁺) medium is described in step 1 and the purification of sperm cells for the assays is described in steps 2-3.

1. Preparation of human tubal fluid (HTF⁺) medium

NOTE: Use volumetric flasks of appropriate sizes, a 1 L measuring cylinder, a magnetic stirrer, salts as listed in **Table 1** and **Table 2**, purified water, a 0.2 μm pore filter, and 50 mL plastic tubes.

1.1 Prepare stock solutions of salts in purified water (**Table 1**).

1.1.1 Add the amount of salt listed in **Table 1** to a volumetric flask of the appropriate size, add purified water to a bit below the desired volume, and mix well using a magnetic stirrer.

1.1.2 When salt is fully dissolved, remove the magnet and fill with purified water to the desired final volume.

1.2 Transfer stock solution to a bottle and store until use.

NOTE: Stock solutions can be kept and reused for longer periods of time: glucose and HEPES at -20 °C and the other stock solutions at room temperature (RT).

1.3 Prepare 1 L of HTF⁺ medium from stock solutions (**Table 2**).

1.3.1 Ensure that all stock solutions are completely dissolved and well mixed before use.

1.3.2 Add the amount of stock solution and salt listed in **Table 2** to a 1 L measuring cylinder and add purified water to 900 mL.

1.3.3 Mix well using a magnetic stirrer and adjust pH to 7.35 with NaOH solution.

1.3.4 Remove the magnet and add purified water to 1,000 mL.

1.4 Sterile filtrate HTF⁺ medium through a 0.2 μm pore filter, aliquot HTF⁺ medium to 50 mL plastic tubes, and store at 4 °C until use.

1.5 Just before running an experiment, add 165 μL of Na-pyruvate to the 50 mL aliquot to get a final Na-pyruvate concentration of 0.33 mM.

NOTE: Human tubal fluid medium can also be obtained from various commercial providers. When using the 4 mM HCO_3^- medium, samples can be incubated with closed lids in an incubator without extra CO_2 in the air without large drifts in pH. If a CO_2 incubator is available, the corresponding amount of CO_2 can be calculated using the Henderson-Hasselbalch equation and used. If using the 25 mM HCO_3^- medium, samples should be incubated with open lids in a CO_2 incubator with air containing a corresponding amount of CO_2 (depends on atmospheric pressure, usually between 5-10%) to avoid a drift in pH. The exact amount of CO_2 can be calculated using the Henderson-Hasselbalch equation.

2. Purification of motile sperm cells via swim-up (Figure 1)

NOTE: Use a clean wide-mouthed plastic container for the semen sample, an incubator, 50 mL plastic tubes, a rack for placing 50 mL plastic tubes at a 45° angle, HTF⁺ medium (prepared in step 1 or obtained from commercial provider), a centrifuge, and human serum albumin (HSA).

2.1 Obtain a freshly donated human semen sample produced through masturbation and ejaculated into a wide-mouthed clean plastic container. Only use semen samples that fulfill the parameters established by the WHO laboratory manual for examination and processing of human semen.

2.2 Incubate the sample at 37°C for 15-30 min to allow it to liquefy.

2.3 Heat up 50 mL of HTF⁺ medium with 4 mM HCO_3^- to 37°C .

2.4 Place clean 50 mL plastic tubes in a rack at a 45° angle (to increase the surface area between liquids). Use 1 tube per mL of raw semen.

2.5 Add 4 mL of the preheated HTF⁺ medium to each of the tubes.

2.6 Carefully pipette 1 mL of the liquefied semen sample to the bottom of each tube containing 4 mL of preheated HTF⁺. Avoid release of air bubbles.

2.7 Close the lids and incubate the tubes at 37°C for 1 h.

2.8 Gently aspirate and transfer as much of the upper fraction (HTF⁺ with motile sperm cells) as possible to a new 15 mL plastic tube, while paying attention that nothing from the bottom fraction (semen with immotile and dead cells) is included. Generally, it is safe to transfer 3.5 mL of the top fraction, without disturbing the bottom fraction. To avoid suction turbulence, cut off the outermost 1-2 mm of the pipette tip at a 45° angle to obtain a larger opening.

2.9 Fill up the 15 mL plastic tube with HTF⁺ to wash cells and centrifuge the tube at 700 x *g* for 10 min at RT.

2.10 Gently aspirate and remove the supernatant and resuspend the sperm cell pellet in 2 mL of HTF⁺.

2.11 Transfer 20 µL of the sperm suspension to two small plastic tubes for assessment of sperm concentration (see step 3).

2.12 Fill up the 15 mL plastic tube with HTF⁺ to wash cells and centrifuge the tube at 700 x *g* for 10 min at RT.

2.13 Gently aspirate and remove the supernatant and resuspend the sperm cell pellet to 10 x 10⁶ cells/mL (based on the cell concentration assessed in step 2.11).

2.13.1 For experiments using un-capacitated sperm cells (routine Ca²⁺-signaling assay)

2.13.1.1 Resuspend cells in HTF⁺ with 4 mM HCO₃⁻.

2.13.1.2 Add 30 µL of human serum albumin (HSA) (100 mg/mL) per mL HTF⁺ to get a final concentration of 3 mg/mL.

2.13.1.3 Close lids and incubate for ≥1 hour at 37 °C.

2.13.2 For experiments using capacitated sperm cells (acrosome reaction assay)

2.13.2.1 Resuspend cells in HTF⁺ with 25 mM HCO₃⁻.

2.13.2.2 Add 30 µL of HSA (100 mg/mL) per mL HTF⁺ to get a final concentration of 3 mg/mL.

2.13.2.3 Attach lids loosely and incubate for ≥3 hours at 37 °C with the corresponding amount of CO₂ (see step 1, usually between 5-10% CO₂).

3. Counting of sperm cells

NOTE: Sperm cells can either be counted manually⁵⁰ or using an image cytometer⁵¹, as described here. Use an image cytometer, a vortexer, S100 buffer, a SP1-cassette, swim-up purified sperm cells (prepared in step 2).

3.1 Dilute a 20 µL aliquot to a factor of 10, 20, 30, 50 or 90 in S100 buffer using a round bottom 2 mL tube according to the expected concentration (evaluated by manual inspection of the pellet in step 2.10). Use the dilution that is closest to the concentration expected (i.e., if a concentration of 15 x 10⁶ sperm cells per mL after swim-up is expected then dilute the 20 µL sperm sample with 380 µL of S100 buffer [dilution factor 20]).

3.2 Mix well for 10 s using a vortex mixer at the maximal 700 rpm, immerse the SP1-cassette into the sample and press the white plunger fully down to aspirate an aliquot of the sample into the cassette.

3.3 Open the image cytometer, place the cassette in the tray and run the assay **Count of PI Stained Human Sperm Cells Assay** according to the applied dilution factor (chosen in step 3.1).

3.4 Repeat step 3.1-3.3 with another 20 μL sample, using a dilution factor closest to the measured concentration obtained from the first 20 μL sample.

3.4 Calculate mean sperm cell concentration from the two measurements.

3.5 Multiply mean sperm cell concentration with the original volume to obtain the total sperm cell count (in step 2, this original volume is 2 mL).

4. Measurement of changes in the free intracellular Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$) using Ca^{2+} -fluorimetry (Figure 2)

NOTE: Use a fluorescence plate reader, 384 multi-well plates, Fluo-4 AM, swim-up purified sperm cells (prepared in step 2), compounds of interest as well as positive and negative controls, a automatic repeater pipette, and a 12-channel pipette.

4.1 Prepare a 2 mM Fluo-4 AM stock by adding 22.7 μL of dimethyl sulfoxide (DMSO) to a vial of 50 μg Fluo-4 AM and mix the tube well.

4.2 Add an aliquot of 700 μL swim-up recovered sperm suspension (capacitated or un-capacitated as outlined in step 2) to a new test tube. 700 μL of sperm sample is the amount needed to analyze one row of 24 wells in the 384-microwell plate (used to test 3 controls and 9 compounds in doublets).

4.3 Add 3.5 μL of Fluo-4 AM stock solution to the 700 μL of sperm suspension to obtain a final concentration of 10 μM Fluo-4 AM.

4.4 Incubate sample for 45 min at 37 $^{\circ}\text{C}$, protected from light.

4.5 Centrifuge sample at 700 x g for 10 min, aspirate and discard the supernatant to remove excess Fluo-4.

4.6 Resuspend stained pellet in HTF⁺ to 5 x 10⁶ cells/mL (i.e., use 2x the volume of cell suspension taken in step 4.2)

4.7 Prepare dilutions of compounds and controls (can be done during the incubation and centrifugation periods in step 4.4 and 4.5, respectively).

4.7.1 Dilute compounds, as well as a negative control (buffer with vehicle) and a positive control (progesterone) in HTF⁺. Dilute compounds and controls to 3x the desired final concentration, as they are diluted 1:3 when they are added to the sperm cells in step 4.16.

4.7.2 Place the tubes with dilutions in a rack with a distance between tubes corresponding to the distance between the pipette tips of the 12-channel pipette used in step 4.16.

4.8 Use an automatic repeater pipette (24 steps of 50 μ L).

4.8.1 Mix Fluo-4 stained sperm sample gently by pipetting the entire amount up and down once.

4.8.2 Add aliquots of 50 μ L to the 24 wells of a row in the 384-microwell plate.

4.9 Place the microwell plate in a fluorescence plate reader at 30 °C and close the drawer.

4.10 Select the appropriate protocol for Fluo-4. Excite fluorescence at 480 nm and record emission at 520 nm. Use the fastest cycle time possible with the setting.

NOTE: Use at least 10 flashes per well and bottom optics, if possible.

4.11 Select a well in the row and adjust gain to a target value of 20%.

4.12 Start the measurement.

4.13 Control the baseline during the first 5 cycles.

4.13.1 If the fluorescent readout is increasing or decreasing over time, stop the experiment, readjust the gain and start the experiment again.

4.13.2 If the baseline differs a lot between individual wells in a row, either the pipetting in step 4.8 has been unprecise or the sample was not mixed well enough before pipetting. Discard the experiment.

4.13.3 If the fluorescent readout is comparable between the wells in the row and steady during the 5 first cycles, continue to the next step.

4.14 After 10 cycles (used for baseline), pause the experiment.

4.15 Eject the drawer with the microwell plate.

4.16 Using a 12-channel pipette (2 steps of 25 μ L), quickly add 25 μ L of the prepared solutions of compounds and controls (from step 4.7) to the 12 well duplicates (24 wells) of a row. Solutions will be diluted 1:3, as the wells already contain 50 μ L of stained sperm sample.

4.17 Continue the measurement as quickly as possible (drawer will automatically close) to avoid missing any fluorescent signals induced by the added compounds and controls. Changes in fluorescence (ΔF) after the addition of compounds and controls will now be captured.

4.18 Run the experiment until fluorescent signals have been recorded from the positive control and the compounds of interest.

4.19 Stop the experiment and export the raw fluorescence data to analyze it as in step 5.

NOTE: Generally Fluo-4 AM has been used as the Ca^{2+} -sensitive fluorophore in the assay, but other Ca^{2+} -sensitive fluorophores could also be used if excitation and emission spectra fit with filters in the fluorescence plate reader. Depending on the choice of fluorophore, make sure that the gain level is set at a “safe” level where the induced fluorescent peaks are within the measuring range of the instrument. This can be tested by adding ionomycin to a single well at a specific gain setting. If this induced a fluorescent signal above the threshold, lower the gain and try again. Some use Pluronic F-127 to aid the loading of Fluo-4^{1,13}, but it has been found that this is not necessary². The maximal number of rows measured simultaneously depends on two factors. 1) The cycle time of the instrument: If the cycle time is too long, induced peaks in $[\text{Ca}^{2+}]_i$ might be missed. Measure a maximum of two rows simultaneously to keep the cycle time low; 2) The speed of pipetting in step 4.16: Using the 12-channel pipette, it is possible to quickly add 12 different solutions to the 12 duplicate wells (24 wells) of 1 or 2 rows (e.g., one row pre-incubated with vehicle and one row pre-incubated with an inhibitor), without missing the induced Ca^{2+} peaks. However, it is not recommended to try to add 24 different solutions to two rows for simultaneous measuring, as pipetting tips will have to be replaced in between the two sequential pipetting steps, whereby induced Ca^{2+} peaks could be missed.

5. Analysis of data from Ca^{2+} -fluorimetry

5.1 Open the raw fluorescence data obtained and exported in step 4.

5.2 Calculate the average from duplicate wells. If large differences exist between duplicates, discard data from the specific wells.

5.3 Define baseline as the 10 first cycles.

5.4 Calculate $\Delta F/F_0$ (%) as the percentage change in fluorescence (ΔF) over time after addition of compounds and controls with respect to the mean basal fluorescence (F_0) during the 10 first cycles (baseline).

5.5 If using the data for generation of dose-response curves, subtract the readout of negative control from the other wells, to remove pipetting artifacts.

6. Measurement of acrosome reaction

NOTE: Use an image cytometer, an incubator, fluorescent dyes: PI, FITC-PSA, and Hoechst-33342, compounds of interest as well as positive and negative controls, an immobilizing solution containing 0.6 M NaHCO_3 and 0.37% (v/v) formaldehyde in distilled water, an A2 slide, and capacitated swim-up purified sperm cells (prepared in step 2).

6.1 Prepare a staining solution containing PI (5 $\mu\text{g}/\text{mL}$), FITC-PSA (50 $\mu\text{g}/\text{mL}$) and Hoechst-33342 (100 $\mu\text{g}/\text{mL}$) in HTF^+ , mix it well using a vortex mixer and protect it from light until use.

6.2 Prepare solutions of compounds and controls at 10x the desired final concentration, as they are diluted 1:10 in step 6.5. Always include a negative (vehicle) control and two positive controls: Progesterone 10 μM final concentration and ionomycin 2 μM final concentration.

6.3 Transfer aliquots of 240 μL of capacitated sperm cells (prepared in step 2) to plastic tubes.

6.4 Add 30 μL of staining solution to each tube. Final concentration of stains will be: 0.5 $\mu\text{g}/\text{mL}$ PI, 5 $\mu\text{g}/\text{mL}$ FITC-PSA, and 10 $\mu\text{g}/\text{mL}$ Hoechst-33342.

6.5 Add 30 μL of solutions with controls or compounds to each tube (1:10 dilution).

6.6 Mix the samples gently by pipetting up and down a few times or mild vortexing. Due to mechanical stress, avoid excessive mixing.

6.7 Incubate at 37 $^{\circ}\text{C}$ for 30 min.

6.8 Prepare new plastic tubes with 100 μL of an immobilizing solution containing 0.6 M NaHCO_3 and 0.37% (v/v) formaldehyde in distilled water, one per test tube.

6.9 After incubation, mix the samples well by pipetting, and add 50 μL of the test sample to a tube with 100 μL of immobilizing solution.

6.10 Before loading the chambers of an A2 slide, mix the sample well by pipetting. Fill the chambers carefully with approximately 30 μL without creating air bubbles.

NOTE: During the incubation, the motile sperm cells tend to aggregate. Cell clumps can disturb the measurements (even though they are excluded). Therefore, a thorough mixing by pipetting after incubation is very important. Likewise, air bubbles in the chambers can disturb the measurements. Therefore, fill the chamber slowly and change the angle of the pipette if edges of the liquid are about to meet and create an air bubble.

6.11 Place the loaded slide on the tray of the image cytometer and close the tray.

6.12 Select the FlexiCyte protocol and press **Run**.

6.12.1 Choose the following settings for the FlexiCyte protocol: Number of analytical channels: 2; Masking channel: UV (LED365), This is the channel used for image segmentation; Channel 1: Blue (LED475), exposure time 3000 ms, emission filter 560/35; Channel 2: Green (LED530), exposure time 500 ms, emission filter 675/75; Minimum number of analyzed cells: 5000; Exclude aggregated cells: Yes.

6.13 Repeat step 6.9-6.12 until all samples have been measured.

7. Analysis of data from image cytometry

7.1 Open data obtained in step 6.

7.2 Create the two following plots to evaluate the measurements.

7.2.1 Create a scatter plot of Hoechst-33342 intensity vs. Hoechst-33342 area on biexponential scales. This plot can be used to gate out Hoechst-33342 positive objects that are either too small or too large to be human sperm cells.

7.2.2 Create a scatter plot of FITC-PSA intensity and PI intensity on biexponential scales. This plot can be used to assess the amount of acrosome reacted sperm cells by use of a quadrant gate that separates the four groups on the plot (**Figure 3**): 1) a PI positive and FITC-PSA positive group: Acrosome reacted nonviable sperm cells; 2) a PI negative and FITC-PSA positive group: Acrosome reacted viable sperm cells; 3) a PI positive and FITC-PSA negative group: Acrosome intact nonviable sperm cells; and 4) a PI negative and FITC-PSA negative group: Acrosome intact viable sperm cells.

REPRESENTATIVE RESULTS:

Representative results from an experiment testing the effect of 4 compounds (A, B, C, and D) together with a positive (progesterone) and negative control on $[Ca^{2+}]_i$ in human sperm using the medium-throughput Ca^{2+} -signaling assay can be seen in **Figure 4a**. In **Figure 4b**, a dose response curve of progesterone is shown, which was derived from peak $\Delta F/F_0$ (%) data induced by serially diluted concentrations of progesterone, tested in another experiment using the medium-throughput Ca^{2+} -signaling assay. The analysis of the data from the Ca^{2+} -signaling assay is explained in step 5. Representative results from an experiment testing the induction of acrosome reaction in capacitated human sperm cells using a negative control (DMSO) or the two positive controls (progesterone and ionomycin) on the medium-throughput acrosome reaction assay can be seen in the top panel of **Figure 3**. Quadrant gated scatter plots from the 3 test conditions are seen. The analysis of the acrosome reaction data is explained in step 7.

FIGURE AND TABLE LEGENDS:

Table 1. Stock solutions for preparation of HTF⁺. Stock solutions can be kept and reused for longer periods of time, Glucose and HEPES at -20 °C and the other stock solutions at room temperature. Ensure that all solutions are completely dissolved and well mixed before use.

Table 2. Preparation of HTF⁺ with 4 or 25 mM HCO₃⁻ from stock solutions of salts (prepared in Table 1). Na-Lactate can be added as syrup, 60 % (w/w), or powder. NaHCO₃ is added as powder.

Figure 1. Swim-up purification of motile human sperm cells. Left: Tube with HTF⁺ medium and an aliquot of liquefied semen sample pipetted below the HTF⁺ medium. Right: After 1 h of swim-up at 37 °C, motile sperm cells have swum up into the HTF⁺ medium. Immotile and dead sperm cells as well as non-sperm cells remain in the semen below the HTF⁺ medium.

Figure 2. Diagram of the medium-throughput Ca²⁺-signaling assay. a) Sperm cells are loaded with a Ca²⁺-sensitive fluorophore, washed and aliquots are plated to the wells of a 384-well plate. b) 384-well plate is positioned in a fluorescence plate reader at 30 °C. c) Fluorescence is recorded before and after addition of compounds, positive control (progesterone) and negative control (buffer with vehicle). Readouts in $\Delta F/F_0$ (%) after addition (arrows) of negative and positive control are illustrated in the bottom of c). Illustration used with permission from Christian Schiffer.

Figure 3. Assessment of acrosome reaction using image cytometry. Top panel: Quadrant gated scatter plots from a negative vehicle control (DMSO), and the positive controls progesterone (Prog, 10 μ M) and ionomycin (Iono, 2 μ M). An increment in cells in the lower right quadrant (acrosome reacted viable cells) is seen when comparing the positive controls to the DMSO control. Bottom panel: Microscopic images of fluorescently labeled sperm cells, including cells from all four groups. BF: Bright field, Hoechst: Hoechst-33342, PSA: Pisum Sativum Agglutinin, PI: Propidium Iodide. Scale bar: 10 μ m. This figure has been modified from Egeberg Palme et al. 2018⁷.

Figure 4. Example of data from Ca²⁺-fluorimetry. a) Changes in [Ca²⁺]_i induced by negative control, progesterone and compound A, B, C, and D. b) Progesterone dose-response curve.

DISCUSSION:

The medium throughput Ca²⁺ signaling assay is based on measurements of fluorescence from single microwells each containing about 250,000 sperm cells. The captured signal is averaged from all individual sperm cells in the well. The assay thus provides no spatial information about where specifically in the sperm cell [Ca²⁺]_i is changed, in how large a proportion of the sperm cells a change in [Ca²⁺]_i takes place, or how heterogeneous the response is between the individual cells. To obtain such information, experiments with single cell resolution (e.g., as described in^{50,52}) must be employed. Another drawback of the technique is the temporal resolution, which is on the order of seconds. Even though the 50 μ L sperm samples are well-mixed when 25 μ L of the solutions are added, the measurements can first be initiated after the drawer with the multi-well plate is back inside the fluorescence plate reader. To obtain higher temporal resolution, other techniques must be employed (e.g., a stopped flow fluorimetry^{13,50}). The advantage of the

medium-throughput Ca^{2+} -signaling assay, compared to single-cell or single-well methods is that the simultaneous measurement of multiple microwells using a microplate reader allows for fast and easy testing of multiple compounds side-by-side with positive and negative controls (**Figure 4**)^{1,2}, easy generation of dose-response curves of individual compounds (**Figure 4**)¹⁻⁵, assessment of additivity^{1,2} and synergism³ for two or more compounds, as well as studies of mode of action of compounds through competitive inhibition experiments and the use of specific pharmacological inhibitors (e.g., CatSper inhibitors¹⁻⁵). Furthermore, by changing the fluorophore, the assay can be employed to examine other intracellular changes (e.g., pH_i ^{1,2}). Finally, more than one fluorophore may be used at once to simultaneously measure multiple intracellular changes. In the future, the medium-throughput Ca^{2+} -signaling assay could be used to screen compounds of interest for effects on Ca^{2+} -signaling in human sperm (e.g., environmental chemicals or drug candidates) to examine the potential adverse effects on human sperm cell function or to investigate a possible role as a contraceptive.

The medium-throughput acrosome reaction assay is based on image cytometry, which constitutes an appealing substitute to assess human acrosome reaction compared to manual evaluation using a fluorescence microscope. Staining patterns of intact or reacting/reacted acrosomes are not always clear, and a great intra-individual variation can be the consequence hereof. With image cytometry, images are acquired and analyzed automatically. The masking channel, in this case Hoechst-33342, defines cells and only fluorescent signals from these specific areas are included in the data analysis. Hence, staining of enucleated bodies containing residual cytoplasm is not included in the data analysis. Furthermore, counting statistics is superior to fluorescence microscopy. To count 200 cells through the oculars of the microscope is a challenging and time-consuming task but with image cytometry the analysis of at least 5,000 cells takes less than a few minutes. However, due to the low magnification of the image cytometer, the ability to evaluate the measurements is lost and one will have to bring the stained sample to the fluorescence microscope. Therefore, the inclusion of controls is of outmost importance to validate the measurements. If the controls do not react as expected, the results from an experiment should be discarded. The protocol described here was inspired by work done by Zoppino et al.⁴⁹, who evaluated human acrosome reaction by flow cytometry. They further described by real time fluorescence microscopy how PSA enters the acrosomal compartment when membrane-fusion pores appear upon induction of acrosome reaction. Once inside the acrosomal compartment, PSA clogs and forms a mark stable for longer periods of time. No fixation or permeabilization is applied in the protocol and therefore the interpretation of the result is clear: PSA inside the acrosomal compartment of a viable sperm cell equals acrosome reaction. As for the Ca^{2+} -signaling assay, the acrosome reaction assay could also be used to assess dose-response curves, inhibition, additivity, and synergism of compounds of interest. In the future, the medium-throughput acrosome reaction assay could also be used to screen compounds of interest for effects on acrosome reaction human sperm (e.g., environmental chemicals or drug candidates) to examine the potential adverse effects on this human sperm cell function or to investigate a possible role as a contraceptive.

ACKNOWLEDGMENTS:

The authors would like to acknowledge the lab of Timo Strünker for supervision of AR and DLE

during their stays at his lab. Furthermore, we would like to thank our colleagues at the Department of Growth and Reproduction, Copenhagen University Hospital, Rigshospitalet for their assistance with setting up these two assays. This work was supported by grants from the Innovation Fund Denmark (grant numbers 005-2010-3 and 14-2013-4).

DISCLOSURES:

The authors have nothing to disclose.

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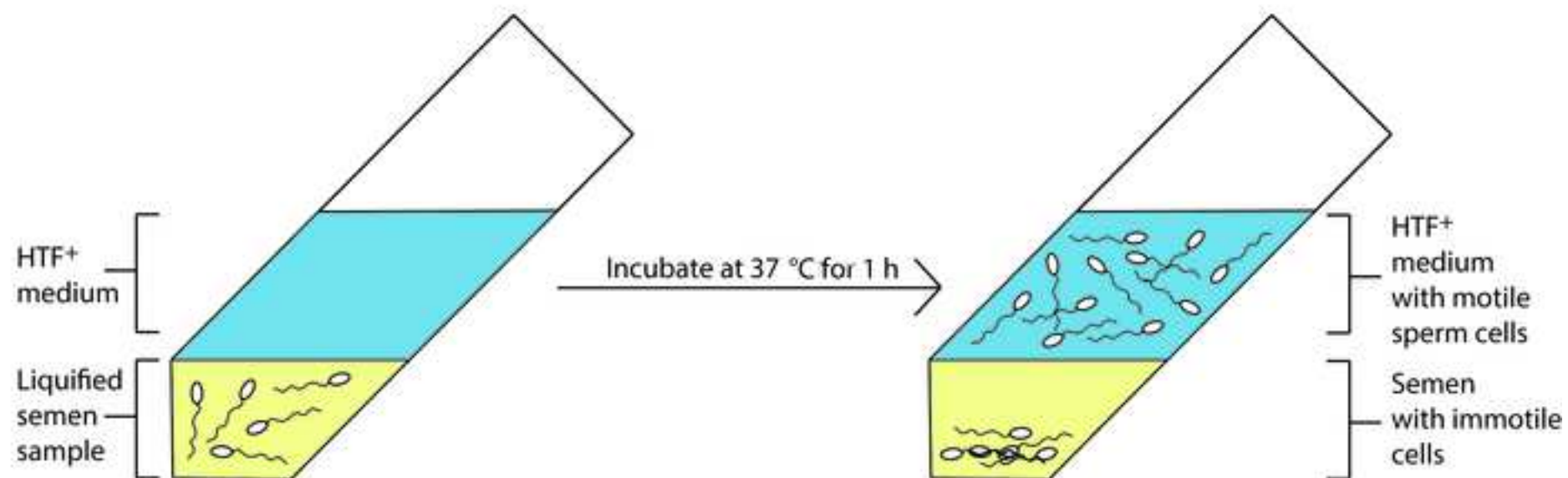
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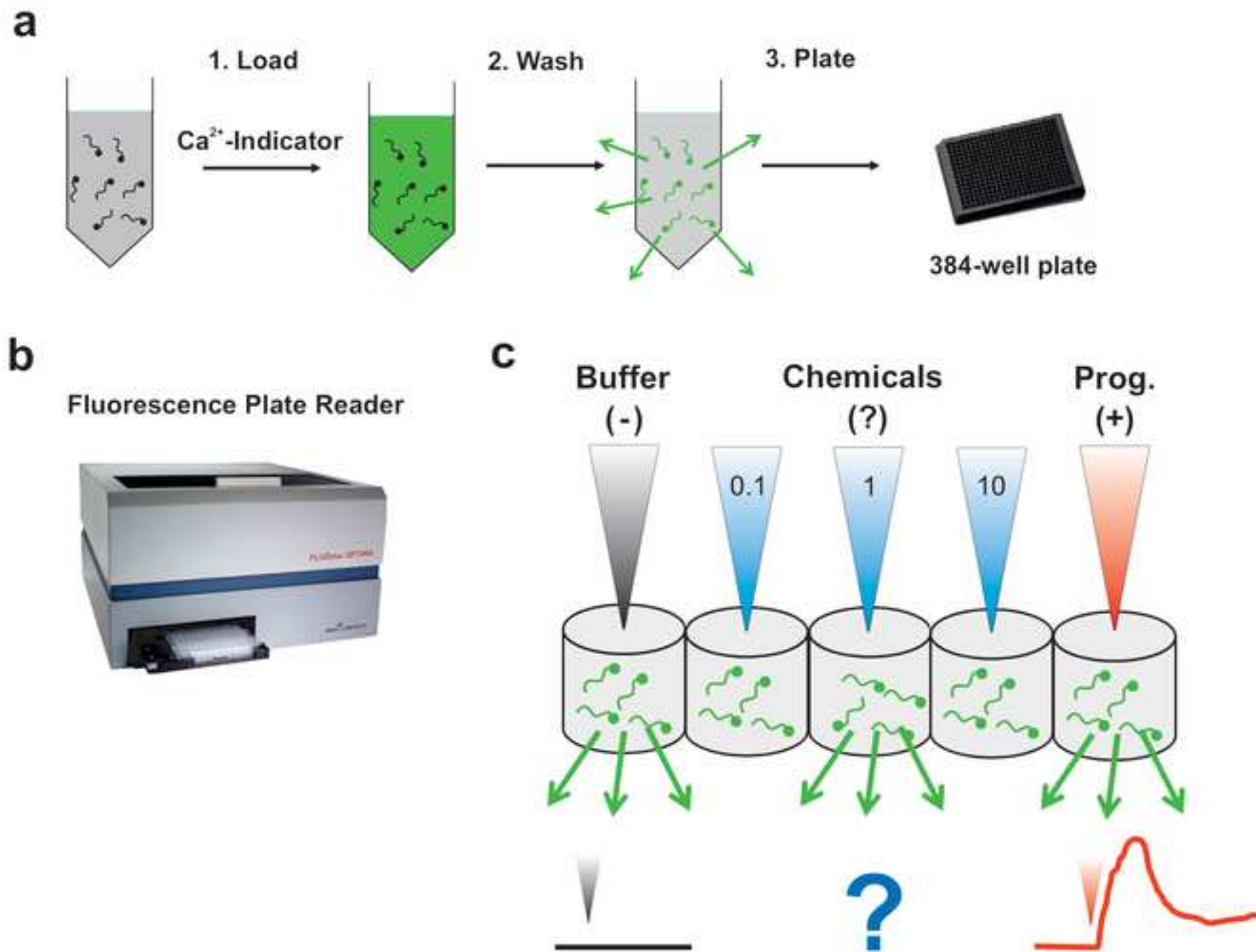
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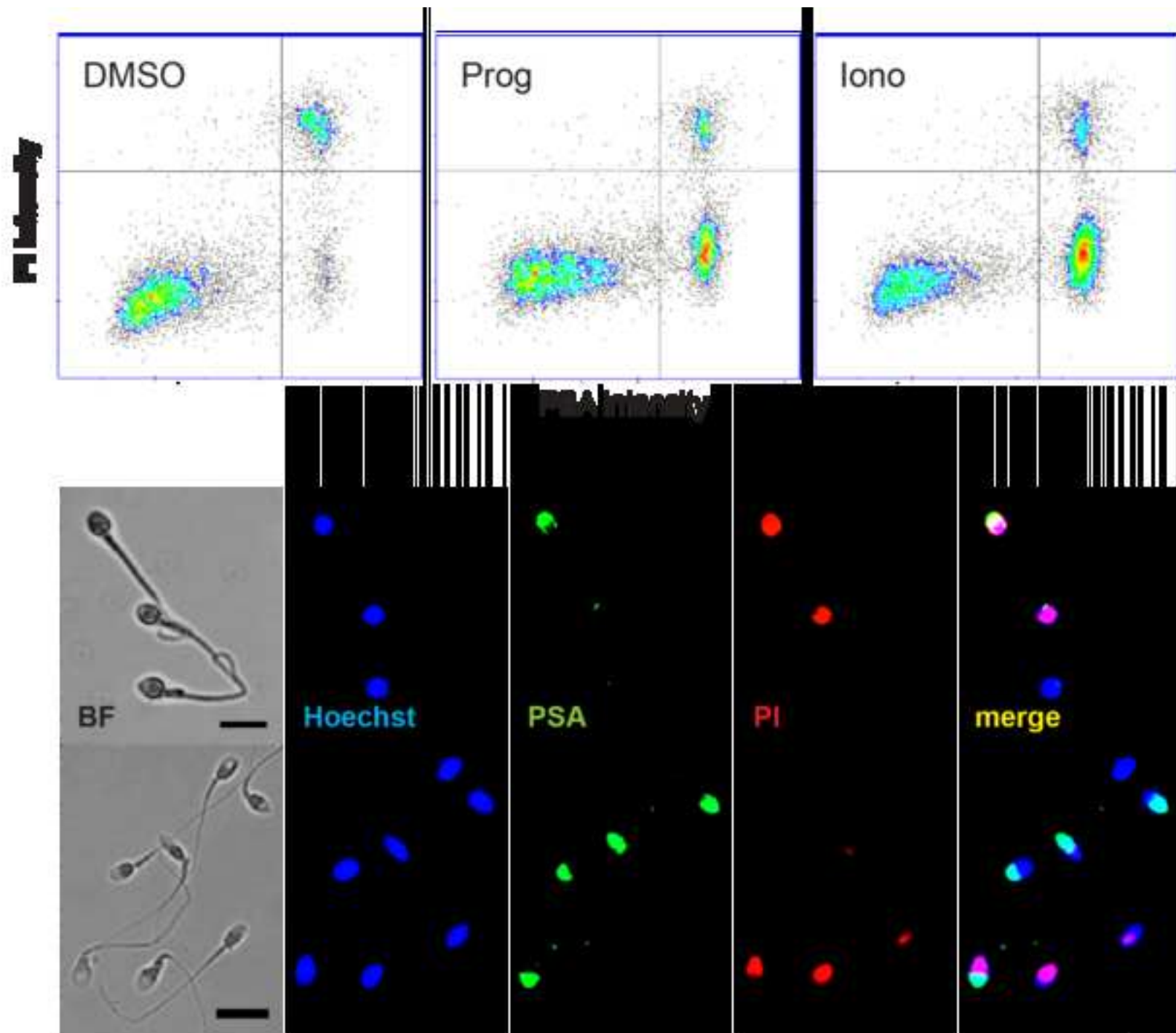
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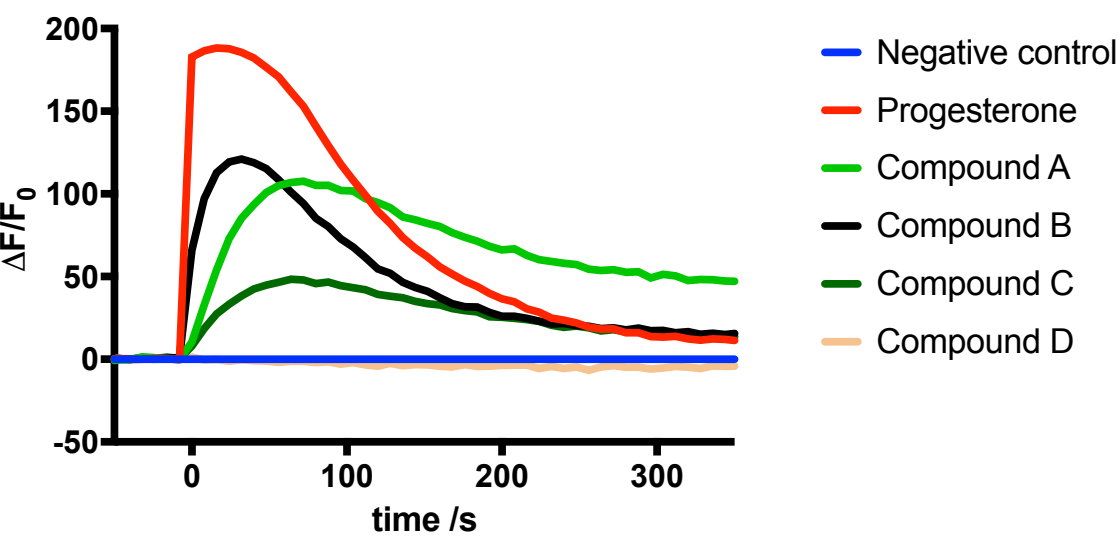
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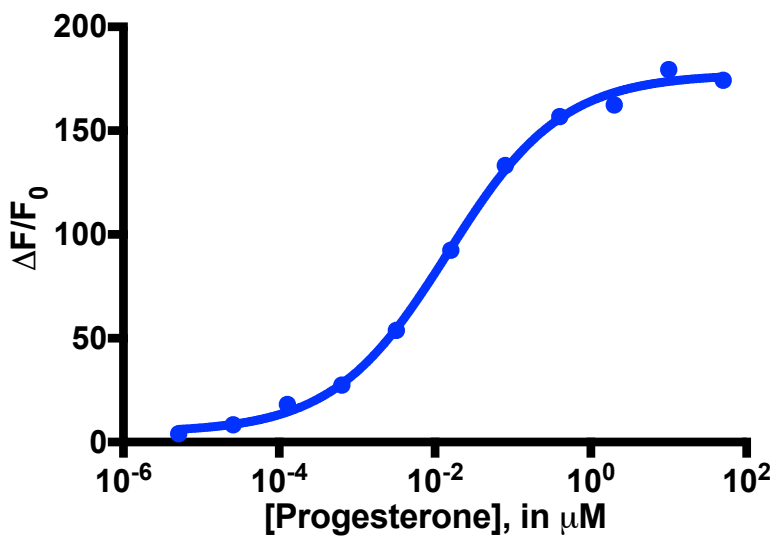




a) Screening of compounds



b)



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 µm pore filter	Thermo Fish	296-4545	
1 L measuring cylinder	Thermo Fish	3662-1000	
1,4 and 2 mL plastic tubes	Eppendorf , Germany	30120086 and 0030120094	
12-channel pipette	Eppendorf , Germany	4861000813	
384 multi-well plates	Greiner Bio-One, Germany	781096	
15 and 50 mL platic tubes	Eppendorf , Germany	0030122151 and 30122178	
A2-slide	ChemoMe tec, Denmark	942-0001	
Automatic repeater pipette	Eppendorf , Germany	4987000010	
CaCl ₂			
Centrifuge			
Clean wide-mouthed plastic container for semen sample			
Dimethyl sulfoxide (DMSO)			
FITC-coupled lectin of Pisum sativum (FITC-PSA)	Sigma- Aldrich, Germany	L0770	

Fluo-4 AM	Thermo Fisher Scientific, USA	F14201	
FLUOstar OMEGA fluorescence mic	BMG Labtech, Germany		
Glucose anhydrous			
HEPES			
Hoechst-33342	ChemoMe tec, Denmark Irvine	910-3015	
Human serum albumin (HSA)	Scientific	9988	For addition to HTF+
Immobilizing solution containing 0.6 M NaHCO ₃ and 0.37% (v/v) formaldehyde in distilled water			
Incubator			
KCl			
KH ₂ PO ₄			
Magnetic stirrer			
MgSO ₄			
Na-Lactate			
NaCl			
NaHCO ₃			
NC-3000 image cytometer	ChemoMe tec, Denmark	970-3003	
Pipettes and pipetting tips			

propidium iodide (PI)	ChemoMe	
Purified water	tec,	
Rack for placing 50 mL plastic tubes in 45° angle	Denmark	910-3002
S100 buffer	ChemoMe	
	tec,	
	Denmark	910-0101
	ChemoMe	
SP1-cassette	tec,	
Volumetric flasks of appropriate sizes	Denmark	941-0006
Vortexer		

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October 5th 2018

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Rebuttal letter:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Has been done.

2. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.

Response: Has been done, primarily in the introduction section.

3. Please remove all headers from Introduction.

Response: Has been done.

4. Please do not use lists in Introduction.

Response: Has been done.

5. Figure 2: Please provide a title for the whole figure in Figure Legend.

Response: Has been done.

6. Please use standard SI unit symbols and prefixes such as μL , mL, L, g, m, etc.

Response: Has been done.

7. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Has been done.

8. All information about equipment or material used in the protocol should be included in Table of Materials.

Response: Has been done for the more specialized equipment. I am assuming, that I don't have to list a specific centrifuge or vortexer, as well as the salts etc.?

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: Has been done.

10. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

Response: Has been done.

11. Line 459: Please ensure that all text is written in imperative tense.

Response: Has been done.

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13. Line 468-470: Please ensure that all text is written in imperative tense.

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Ps. The Editorial Manager was prompting me to upload a figure and the ALA again, I am not sure why. There are no changes in either of these files.