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Measuring intracellular Ca changes in human sperm using four techniques: fluorometry, stopped flow fluorometry, flow cytometry and single cell imaging --Manuscript Draft--

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Dear Dr. Baker:

We would like to thank you for the opportunity to submit our paper: Measuring intracellular [Ca²⁺] changes in human sperm using four techniques: fluorometry, stopped flow fluorometry, flow cytometry and single cell imaging to the Journal of Visualized Experiments. We are grateful for the time the reviewers invested in reading our manuscript and for their valuable comments and suggestions. Please find below a detailed response to every issue raised by the reviewers, and note that all the corresponding modifications are indicated in blue font in the manuscript. We hope that this improved version is suitable for publication.

Sincerely,

Dr. Claudia L. Treviño

Measuring intracellular Ca^{2+} changes in human sperm using four techniques: fluorometry, stopped flow fluorometry, flow cytometry and single cell imaging

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Short Abstract

Intracellular Ca^{2+} dynamics are very important in sperm physiology and Ca^{2+} -sensitive fluorescent dyes constitute a versatile tool to study them. Population experiments (fluorometry and stopped flow fluorometry) and single cell experiments (flow cytometry and single cell imaging) are used to track spatio-temporal $[\text{Ca}^{2+}]$ changes in human sperm cells.

Abstract

Spermatozoa are male reproductive cells especially designed to reach, recognize and fuse with the egg. To perform these tasks, sperm cells must be prepared to face a constantly changing environment and to overcome several physical barriers. Being in essence transcriptionally and translationally silent, these motile cells rely profoundly on diverse signaling mechanisms to orient themselves and swim in a directed fashion, and to contend with challenging environmental conditions during their journey to find the egg. In particular, Ca^{2+} -mediated signaling is pivotal for several sperm functions: activation of motility, capacitation (a complex process that prepares sperm for the acrosome reaction) and the acrosome reaction (an exocytotic event that allows sperm-egg fusion). The use of fluorescent dyes to track intracellular fluctuations of this ion is of remarkable importance due to their ease of application, sensitivity, and versatility of detection. Using one single dye-loading protocol we utilize four different fluorometric techniques to monitor sperm Ca^{2+} dynamics. Each technique provides distinct information that enables spatial and/or temporal resolution, generating data both at single cell and cell population levels.

Introduction

Ca^{2+} is a universal second messenger of signal transduction pathways in eukaryotic cells. Intracellular Ca^{2+} (Ca^{2+}_i) participates in the regulation of many fundamental physiological processes in both excitable and non-excitable cells. The importance and universality of Ca^{2+} as second messenger during signal transduction events is derived from its spatio-temporal versatility in the transmission of information within the cell. While Ca^{2+} cannot be synthesized *de novo* or degraded within the cell, its intracellular concentration ($[\text{Ca}^{2+}]_i$) is maintained within very strict limits through different cellular mechanisms that continuously buffer, sequester, compartmentalize, and/or accumulate Ca^{2+} . Changes in the concentration of this ion can occur in

highly localized areas within the cell ¹, and deciphering such fluctuations is essential for gaining a deeper understanding of (1) their role in the signaling mechanism, (2) their physiological significance, and (3) general mechanisms of cell signaling. Ca^{2+} -mediated signaling is of particular importance in sperm physiology ². Sperm motility is one of the most important functions for fertilization success, and in fact, several sperm motility defects can cause sterility ³⁻⁵. The importance of Ca^{2+} in flagellar movement has been long recognized ⁶; however, the mechanism of how Ca^{2+} controls the specific form of flagellar bending is not fully understood.

Before fusing with the egg, spermatozoa must undergo capacitation, a complex process dependent on sperm residence inside the female tract. During capacitation, the sperm membrane's lipid architecture and organization are modified, mainly as a result of cholesterol removal from the plasma membrane. Additionally, several proteins are tyrosine-phosphorylated ⁷. Importantly, during capacitation there is an increase in intracellular pH (pH_i) and in $[\text{Ca}^{2+}]_i$, and the membrane potential hyperpolarizes in some species ². Capacitation only takes place in a subpopulation of spermatozoa (20-40%), and the mechanisms involved in all these cellular changes are far from clear. It is generally accepted that only a subpopulation of capacitated sperm undergo the acrosome reaction (AR) when exposed to physiological inductors. The AR is also a Ca^{2+} -regulated event required for fertilization in all species possessing an acrosome (specialized organelle with outer and inner membranes). During this process the outer acrosomal membrane fuses with the sperm's plasma membrane, releasing hydrolytic enzymes that allow the sperm cell to penetrate the glyco-proteinaceous matrix surrounding the egg (zona pellucida, or ZP). The AR also exposes a new fusogenic sperm cell surface that interacts with the egg plasma membrane for the final fusion of both gametes. There are several cellular ligands that induce the AR, progesterone being one of the most studied among them.

In this work we present four different techniques involving the use of a Ca^{2+} -sensitive fluorescent dye to measure $[\text{Ca}^{2+}]_i$ changes in human sperm triggered by progesterone (except for flow cytometry, in which we measured the $[\text{Ca}^{2+}]_i$ increase induced during the *in vitro* capacitation process). In this particular case we used Fluo-3 AM (Life Technologies, Grand Island, NY), a membrane-permeable dye with a $K_d = 325 \text{ nM}$. *In vitro* we monitored fluorescence changes as a function of time with three of the methodologies, and with the fourth technique we measured fluorescence values at a single given point in time. These different approaches complement each other, since altogether they provide spatial and temporal resolution at both the single cell and cell population levels.

Cell Population or Bulk Experiments

Bulk techniques are extensively used not only because the instruments they require are readily available, but also because they are simple, well established, and allow for the averaging of information from measurements performed on millions of cells in a single experiment.

Technique #1. Conventional Fluorometry

This technique monitors changes in fluorescence as a function of time; the experiments are performed in glass cuvettes with sample volumes ranging from 200 to 1000 μL . Proper mixing of added reagents requires magnetic stirring, and therefore the temporal resolution obtained is in the order of seconds. The typical cell concentration range of the samples analyzed is 10^5 - 10^8 cells/mL.

Technique #2. Stopped Flow Fluorometry

This technique also monitors changes in fluorescence as a function of time, but the reagents are rapidly mixed together (using pressure) into a recording cuvette containing a very small sample volume (ranging from 25-100 μL). Therefore, homogenization of reagents is instantaneous, enabling a high temporal resolution in the order of milliseconds. Analysis of the resulting fluorescence vs. time traces are suitable for determining reaction rates, elucidating the complexity of the reaction mechanism, obtaining information on short-lived reaction intermediates, etc. The common cell concentration range of the samples analyzed is 10^5 - 10^7 cells/mL.

Single Cell Experiments

Bulk experiments report the average behavior of a large number of cells; however, a population may frequently exhibit heterogeneous properties that are overlooked during such type of measurements. Single cell techniques are thus used to complement the information obtained with cell population experiments.

Technique #3. Flow Cytometry

Despite the importance of the information arising from single cell measurements, it is important to analyze a large number of cells in order to prevent the erroneous extrapolation of cell-specific properties to an entire population. For this reason, high-throughput techniques are favored and the most popular method is flow cytometry, in which 10,000 cells per condition are conventionally analyzed. This method enables multi-parametric analysis of heterogeneous populations as it categorizes cells according to their size (forward scatter (FSC)), granularity (side scatter (SSC)) and fluorescence intensity (specific labeling with an antibody, viability marker, etc.), thus providing information on the parameters' distribution for a group of cells. Flow cytometry provides instant rather than time-dependent information⁸. Forward and side scatter values are also useful for selecting a gate that includes cells but discriminates cellular debris, dust, etc. For fluorescence measurements, negative and positive fluorescence controls must also be included. If more than one fluorescence channel is used, a process known as compensation must be performed (for details see

http://www.bdbiosciences.com/support/resources/protocols/setting_compensation.jsp).

Compensation allows for spectral overlap discrimination among fluorophores. Flow cytometry also allows discrimination of dead cells, generally by means of propidium iodide staining.

Technique #4. Single Cell Imaging

Microscopy is another common method to study single cell behavior; it is well suited for time-dependent studies and it also provides spatial resolution. A major drawback is that high-throughput analysis is only in its infancy at the present time⁹.

Protocol Text

In this paper we report the use of the four aforementioned techniques to measure $[\text{Ca}^{2+}]_i$ changes in human sperm cells. We used progesterone to trigger a Ca^{2+} response, as it is well established that this steroid produces a transient $[\text{Ca}^{2+}]_i$ increase in spermatozoa. Particularly, in human sperm, progesterone directly activates a Ca^{2+} channel (namely CatSper) expressed exclusively in the plasma membrane of sperm cells^{10,11}. We also measured resting $[\text{Ca}^{2+}]_i$ before and after capacitation given that it is also widely accepted that an increase in $[\text{Ca}^{2+}]_i$ occurs during

capacitation. For techniques requiring a positive control we used a Ca^{2+} ionophore –ionomycin– to induce maximal Ca^{2+} uptake into the cell, and thus, maximal fluorescence response; for the minimal fluorescence value, we used Mn^{2+} to quench fluorescence.

1. Sperm sample preparation by the swim-up method (see Figure 1)

Use only ejaculated samples (obtained by masturbation) whose characteristics fulfill the parameters established by the latest edition of the World Health Organization laboratory manual (available at http://whqlibdoc.who.int/publications/2010/9789241547789_eng.pdf) for the examination and processing of human semen.

- 1.1** Obtain the semen sample inside a sterile container and place it (with loosened cap) inside an incubator at 37°C and CO_2 5%/air 95% during 30 minutes. This step is for sample liquefaction.
- 1.2** Place 500 μL aliquots of the liquefied semen sample on the bottom of clean glass test tubes (1.0 x 7.5 cm). Approximately eight test tubes are needed for an average size sample (4 mL).
- 1.3** Carefully layer 1 mL of Ham's F-10 medium (supplemented with 2 mM CaCl_2 and 5mg/mL bovine serum albumin to promote capacitation *in vitro*) on top of each semen aliquot (See Figure 1). *TIP:* Touch the wall of the tube with the tip of the micropipette, and gently dispense the medium above the sample. It is crucial to do it slowly as the mixing of both layers (sample and medium) must be avoided.
- 1.4** Carefully lean the tubes to a 30° angle approximately. This will increase surface area between the two liquids, thus enhancing the displacement (swim-up) of human cells from the sample to the medium during incubation.
- 1.5** Place the group of leaned test tubes inside an incubator at 37°C and CO_2 5%/air 95% for 1 hour.
- 1.6** Using a micropipette carefully remove the upper 700 μL of HAM's F-10 medium (now containing motile spermatozoa) from each tube and pool all the collected samples into a single clean glass tube (1.0 x 7.5 cm; for larger volumes use a 15 mL Falcon tube), avoiding bubble formation. Place 10 μL of pooled sample on the optical flat glass of a Makler Counting Chamber base, and then place the cover glass (once the cover is in place, avoid lifting or covering again to maintain the uniform spread of sperm sample). Make sure to avoid bubble formation inside the chamber as this would result on an inaccurate cell count.
- 1.7** Observe under a compound microscope (the use of a 20X objective is recommended). The cover glass of the Makler Counting Chamber has a big square composed of 100 smaller squares (i.e., a 10 by 10 grid). Count the cells in any strip of 10 squares. This number represents their concentration in millions of cells/mL. Repeat the count in two additional 10-square strips, and calculate the average of the three counts. *NOTE:* If a Makler Counting chamber (which is especially designed to count sperm cells) is not available, any hemocytometer chamber may be used.
- 1.8** Adjust the sample's final concentration to 1×10^7 cells/mL in supplemented Ham's F-10 medium. When required, incubate the sample at 37° C and CO_2 5%/Air 95% for 5 hours to promote capacitation.

2. Fluorescent dye loading for Ca^{2+} measurements

There are several fluorescent dyes available to measure intracellular Ca^{2+} ; the appropriate one must be selected according to its K_d , and its emission and excitation wavelengths (for qualitative and quantitative measurements, single and double emission and excitation wavelengths, respectively, must be used) (visit <http://es-mx.invitrogen.com/search/global/searchAction.action?query=ion+indicators&resultPage=1&resultsPerPage=15> for more information). For the present qualitative application we used Fluo-3 AM, a cell-permeant dye with a $K_d = 325$ nM, and single emission and excitation wavelengths of 506/526 nm, respectively ¹².

2.1 Prepare 50 μL of a 1 mM Fluo-3 AM stock solution by dissolving the content of one 50- μg dye vial ($\text{MW} = 1130$ g/mol) in 44 μL of anhydrous DMSO.

2.2 Using a 1.5 mL microfuge tube mix the required volume of sperm suspension (see required amount for each specific technique below) with enough 1 mM Fluo-3 AM stock solution to obtain a final concentration of 2 μM Fluo-3 AM (i.e. 1 μL of stock Fluo-3 AM is added for every 500 μL of sperm suspension).

2.3 Incubate for 30 minutes at 37°C and protected from light.

2.4 Centrifuge the tube at 750 g for 5 min using a microcentrifuge, aspirate and discard the supernatant, and resuspend the pellet in the appropriate volume (see required concentration for each specific technique below) of Human Sperm Medium (HSM; mM: 120 NaCl, 15 NaHCO_3 , 4 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 Na lactate, 5 D-glucose, 1 Na pyruvate, pH = 7.4). *NOTE:* The formation of a cloud rather than a pellet indicates that the cells are in good condition.

2.5 The cells are now loaded with the dye; they remain viable (kept at 37°C and protected from light) for approximately two hours, and may be used in any of the following techniques.

3. Technique #1. Conventional Fluorometry (average information from a large cell population)

Equipment: For our sperm population $[\text{Ca}^{2+}]_i$ measurements we use an SLM Aminco spectrofluorometer operated by Olis software (Bogart, GA, USA) with magnetic stirrer control (SIM Aminco), and coupled to a Blue LED (Luxeon Star LXHL-LB3C, from LUMILEDS) and a 465-505 nm band-pass filter (Chroma Technology Corp.) for Fluo-3 AM excitation. The LED is controlled by a custom-built power supply (700 mA). Emission light is measured by setting the emission wavelength (λ_{Em}) to 525 nm on the spectrofluorometer's monochromator.

3.1. Place 570 μL of HSM and 30 μL of sperm cell suspension (previously loaded with Fluo-3 AM and resuspended in HSM to obtain 1×10^8 cells/mL) in a flat bottom glass tube (I.D. 8 x 50 mm). Place a magnetic stir bar inside the tube and insert the tube into the reading chamber of the spectrofluorometer (preheated to 37°C), stir the sample during all the acquisition time.

3.2. Start the experiment using the equipment's software (Olis software in this case) and proceed to acquire fluorescence values at a frequency of 0.5 Hz during 600 s. Apply the desired test compounds by injection of the appropriate volume from a stock solution (generally 100X more concentrated than the desired final concentration) using a Hamilton micro-syringe as follows:

3.2.1 Acquire basal fluorescence for 30 s.

3.2.2 Add 4 μM progesterone (Pg).

3.2.3 At 100 s add 20 μM ionomycin (as a positive control, to obtain the maximum fluorescence value).

- 3.4 Run a negative control by repeating steps 3.1 to 3.2.3 above, but adding instead of Pg only the solvent used to dissolve it (HSM with 0.01% anhydrous DMSO).
- 3.5. Export raw fluorescence intensity values to Microsoft Excel and normalize them using the following equation: $(F/F_0) - 1$. Where F is the fluorescence intensity measured at any given time (t), and F₀ is the mean basal fluorescence taken during the initial 30 s. Plot the total series of $(F/F_0) - 1$ values vs. time (Figure 2A). Measure the difference between the fluorescence intensity values before and after the addition of the test compounds (ΔF), plot them on a bar graph and process the data applying the appropriate statistical analysis methods (Figure 2B).

4. Technique #2. Stopped Flow Fluorometry (information with high temporal resolution from a large cell population)

Equipment: Intracellular $[Ca^{2+}]$ changes are measured with high temporal resolution using a SFM-20 stopped-flow mixer coupled to a MOS-200 rapid kinetics optical system, both from BioLogic science instruments (Grenoble, France). All data are analyzed with Bio-Kine32 software from the same company.

- 4.1 Set the appropriate conditions in the equipment; the illumination source should be turned on at least 15 minutes before starting the experiment; adjust excitation and emission filters, adjust the photomultiplier to a voltage value within the range established by the stopped-flow manufacturer, and set the bath temperature at 37° C.
- 4.2 Fill one of the instrument's syringes with 1 mL of Fluo-3 AM-loaded sperm cells (1×10^7 cells/mL) and the second syringe with 1 mL of the compound to be tested, either HSM (negative control), 10 μ M ionomycin (positive control) or 10 μ M Pg dissolved in HSM. *Note:* In this step it is crucial to avoid bubble formation while drawing the liquids into the syringes.
- 4.3 Lift both instrument pistons until they touch the tip of the syringe plungers.
- 4.4 Set the flow rate to the minimum value that will provide a measurable response in order to minimize cell damage. The flow rate we use in the SFM-20 system is 1 mL/s¹³.
- 4.5 Set the frequency (in this case 10 ms) and the total sampling time (in this case 50 s).
- 4.6 Trigger the mixing of reagents. *NOTE:* While one single trigger at a time may be made manually, a set of automatic consecutive triggers may be pre-programmed as well.
- 4.7 The trace of raw fluorescence (arbitrary units) vs. time is displayed on the computer screen.
- 4.8 The mixing of reagents *per se* will generate a trace that is not a straight line. Thus, in order to obtain the actual $[Ca^{2+}]$ change derived from a stimulus, the control trace obtained from mixing cells with medium (negative control) must be subtracted from each one of the experimental traces. Analyze data as required; some kinetics parameters may also be obtained with the Bio-Kine32 acquisition software. *Raw traces without subtraction are shown in Supplementary Figure 1 for comparison.*
- 4.9 To change the reagent in the test compound syringe, clean it out thoroughly with distilled water. Then fill the syringe to its maximum volume with distilled water, place it in the corresponding piston of the stopped-flow fluorometer and push the water through the internal mechanism (the rinse water must be directed to the waste container). Repeat this step twice more.
- 4.10 Repeat steps 4.2 to 4.9, filling the second syringe with the next desired test compound.
- 4.11 At the end of the experiment, rinse the entire equipment with distilled water, completely draining the water from the internal hoses.

5. Technique #3. Flow Cytometry (single cell information obtained from a large number of cells).

Equipment: This technique allows the simultaneous measurement of several parameters in a single moment in time, but unlike the previous techniques, it does not measure changes over time; rather it provides the parameter values at the time of measurement. Therefore, instead of adding Pg to trigger the response, in this case we measured intracellular Ca^{2+} levels in sperm cells before and after inducing capacitation. We used a FACSCanto™ Cytometer (Becton Dickinson) and data were analyzed with FlowJo software (Tree Star 9.3.3).

5.1 Prepare the experimental samples in cytometer tubes by placing 500 μL of cell suspension (4×10^6 cells/mL) per tube under each condition to be tested (in this case, ten conditions; see Table 1). Collect fluorescence data from 10,000 events per sample.

5.2 To set up an experiment use the equipment Software to:

5.2.1 Create a new: folder, experiment, specimen and number of tubes.

5.2.2 Select appropriate cytometer settings for Fluo-3 AM (use FITC -Fluorescein isothiocyanate- filter) and PI (use PI -Propidium Iodide- filter).

5.3 Run the unstained control tubes 1 and 2 in the cytometer. Collect FSC and SSC data to verify that threshold settings are appropriate and to create the corresponding gate in order to discriminate debris from cells.

5.4 To create compensation controls, run the following control samples, collecting auto and maximum fluorescence data (PI and FITC channels) (*NOTE:* this task is usually performed by the equipment's technician):

- ✓ Unstained cells (tubes 1 and 2).
- ✓ Cells loaded with Fluo-3 AM (2 μM) (tubes 3 and 4).
- ✓ Dead cells (spermatozoa suspended in 0.1% Triton X-100 in HSM for 10 min at room temperature) stained with PI (1.2 μM PI; i.e. 0.25 μL of 2.4 mM PI is added to 500 μL of sperm suspension) during 30 minutes at 37°C, protected from light (tubes 5 and 6).
- View recorded data and select the gate for the desired populations.
- Adjust the gate and select “Apply” to All Compensation Controls.
- Select experiment > compensation setup > calculate compensation.
- Rename the compensation setup and link & save.

5.5 Run all experimental tubes (in this case, tubes 7-10). At the end, export all data to the software available for analysis (see step 5.6).

5.6 Analyze the results of each experiment using the equipment's software, the commercially available FlowJo software or Cytobank free software (<http://www.cytobank.org/>).

6. Technique #4. Single Cell Imaging (single cell information with high spatial resolution).

Equipment: Custom-built Imaging set-up. Our imaging set-up is composed of an inverted Nikon Diaphot 300 microscope equipped with a temperature controller (Medical System Corp., Greenvale, N.Y.), a Nikon PlanApo 60X (1.4 NA oil immersion) objective. Fluorescence illumination is provided by a Luxeon V Star Lambertian Cyan LED part # LXHL-LE5C (Lumileds Lighting LLC, San Jose, CA) attached to a custom-built stroboscopic control box. The LED was mounted into a FlashCube40 assembly with dichroic mirror M40-DC400 (Rapp Opto Electronic, Hamburg, Germany) (bandwidths: excitation 450–490 nm, dichroic mirror 505 nm, and emission 520–560 nm). LED output was synchronized to the Exposure Out signal of a Cool Snap CCD camera via the control box to produce a single flash of 2 ms duration per individual

exposure. The camera exposure time was set equivalent to the flash duration (2 ms). Images are collected every 250 ms (or may be adjusted according to the desired temporal resolution) using IQ software (Andor Bioimaging, Wilmington, NC).

- 6.1 Prepare round cover slips (diameter = 25 mm) by applying a 5- μ L drop of poly-L-lysine solution (0.01 % w/v) on the center. Let it stand for at least 1 hour (it may dry). Using a squirt bottle rinse treated area with water before use. This procedure will allow sperm cells to adhere to the cover slip from their head, while their flagellum can still move.
- 6.2 Prepare the compounds to be tested by dissolving them in HSM according to Table 2. Compounds are added sequentially into the same recording chamber, making sure to always add the same volume, and to adjust the concentration of the stock solution taking into consideration the dilution it will have when mixed with the volume already present in the chamber (as indicated in Table 2). Keep all test solutions in a bath at 37°C until they are used.
- 6.3 Assemble the cover slip inside the recording chamber and place 10 μ L of Fluo-3 AM-loaded cells (1×10^7 cells/mL) in the center. Cover the cells with 200 μ L of pre-warmed HSM.
- 6.4 Place the chamber on the stage of the microscope pre-heated to 37°C, view the cells (using phase contrast) and select an area for imaging. It is important to select an area where cell density is appropriate (see Figure 5A); too many cells make analysis difficult due to overlapping signals. *NOTE:* Cells should be firmly attached to the cover slip by their head but exhibiting flagellar movement, which confirms viability.
- 6.5 Acquire fluorescence images in live mode to adjust focus and brightness.
- 6.6 Start the experiment by activating the time-series image acquisition software (IQ in this case). Typically four images are acquired per second with illumination of 2 ms per image.
- 6.7 Use a micropipette to carefully add (drop-wise) the test compound (Pg in this case), continue image acquisition as required and perform two sequential control additions into the same chamber: (1) 20 μ M ionomycin to obtain maximum fluorescence and (2) 5 mM MnCl_2 to obtain minimum fluorescence. Alternatively, compounds may be added using a perfusion chamber which offers the advantages of enabling stimulus removal, and the ability to uniformly bathe the cells with the compound. At the same time, it does have the disadvantages of requiring larger quantities of solution, and of making temperature control more problematic.
- 6.8 Repeat acquisition in a new chamber with every desired test compound.
- 6.9 Perform image analysis online using the equipment's software, or offline using either IQ Software or Image J freeware. Draw the regions of interest (ROIs) around each cell (or part of cell) and also select a cell-free area (for automatic background subtraction by the software). A time-fluorescence intensity series is then obtained for each ROI and these data may be exported to Microsoft Excel for further analysis. We normalize fluorescence intensity values using the following equation: $(F/F_0) - 1$. Where F is the fluorescence intensity measured at any given time (t) and F_0 is the mean fluorescence taken during the initial 30 seconds. Plot the total series of $(F/F_0) - 1$ vs. time (Figure 5B). Values may also be normalized using the fluorescence value obtained after ionomycin addition as 100%.
- 6.10 Image Analysis may alternatively be performed using Image J free Software.

Representative Results

Technique #1. Conventional Fluorometry.

Progesterone is one of the known AR inducers and, as expected, it does provoke a transient $[Ca^{2+}]_i$ increase in human sperm (shown in Figure 2). Addition of a calcium ionophore (ionomycin) causes the maximum $[Ca^{2+}]_i$ increase, which does not return to basal levels.

Technique #2. Stopped Flow Fluorometry.

The progesterone-induced $[Ca^{2+}]_i$ increase was measured as before (conventional fluorometry), but this time with greater temporal resolution; in this case the frequency of acquisition was 0.1 Hz. As shown in Figure 3, both progesterone (transient, red line) and ionomycin (sustained, blue line) caused a very fast $[Ca^{2+}]_i$ increase. The absence of a delay in the progesterone-induced $[Ca^{2+}]_i$ increase is consistent with previous reports suggesting that progesterone directly activates the Ca^{2+} channel CatSper, without intermediate signaling^{10,14}.

Technique #3. Flow Cytometry.

$[Ca^{2+}]_i$ was measured in capacitated and non-capacitated human sperm. As previously reported in mouse¹⁵, bovine sperm¹⁶ and human sperm¹⁷, we also observed increased $[Ca^{2+}]_i$ in capacitated compared to non-capacitated human sperm. Baldi et al. (1991)¹⁷ reported higher basal $[Ca^{2+}]_i$ in capacitated than in non-capacitated human sperm using conventional fluorometry. In this work we used flow cytometry to measure $[Ca^{2+}]_i$ before and after *in vitro* capacitation. Flow cytometry enables us to see that the distribution of fluorescence values for capacitated sperm (Figure 4D, blue trace) is shifted to higher values compared to non-capacitated sperm (Figure 4D, red trace). The fluorescence values for each individual cell can be observed in the two-dimensional dot plots shown in Figure 4G; importantly, the signal arising from dead cells (15% approximately) can be eliminated (Figure 4G, upper quadrants).

Technique #4. Single Cell Imaging.

The progesterone-induced $[Ca^{2+}]_i$ change was measured in single sperm cells. Progesterone addition causes an increment in $[Ca^{2+}]_i$ both in the sperm head and in the flagellum. As observed in population experiments, single cell analysis revealed a transient and a sustained increase for progesterone and ionomycin, respectively.

Discussion

Intracellular signaling is vital for most cellular activities; Ca^{2+} is a ubiquitous messenger that accompanies mammalian cells throughout their entire lifespan, from their origin at fertilization, to the end of their life cycle. In response to different stimuli, $[Ca^{2+}]_i$ increases, oscillates and decreases with spatio-temporal codification; accordingly, diverse processes are activated, modulated or terminated by Ca^{2+} -encoded messages. Intracellular Ca^{2+} dynamics are very important in sperm physiology, as this ion is involved in a complex orchestration of signal transduction processes. Knowledge of such Ca^{2+} fluctuations can help decipher the molecular identity of the Ca^{2+} channels involved, and their specific roles in distinct signaling cascades. Ca^{2+} -sensitive fluorescent dyes constitute a powerful tool to study them, enabling highly specific and sensitive Ca^{2+} measurements that may be used to track concentration changes in living cells. In this paper, cell population experiments (conventional and stopped flow fluorometry) and single cell experiments (flow cytometry and single cell imaging) are used either to follow spatio-

temporal $[Ca^{2+}]$ changes in human sperm cells upon a progesterone stimulus, or to estimate Ca^{2+} resting levels before and after *in vitro* capacitation.

Conventional fluorometry is a widespread technique because the required instrumentation is readily available in most laboratories, it is easy to perform, and it can rapidly inform whether there is a $[Ca^{2+}]_i$ increment upon a specific stimulus. Stopped flow fluorometry, although requiring more sophisticated equipment, provides high temporal resolution. For example, Kaupp and coworkers determined that upon progesterone stimuli, sperm $[Ca^{2+}]_i$ rises immediately with no delay¹⁰; this fact, in conjunction with additional experimental evidence¹⁴, lead them to propose that progesterone directly activates a Ca^{2+} channel (CatSper), ruling out the involvement of a signaling cascade or of an additional progesterone receptor, at least during the initial progesterone-induced increment. Progesterone induces a transient increase followed by a smaller and slower increment; therefore, more than one Ca^{2+} entry mechanism may be involved in the overall response.

Flow cytometry is a very powerful technique but it requires expensive instrumentation. When available, this method is of remarkable importance, as it simultaneously measures several parameters, on a large number of cells, and in a relatively short time. Importantly, it allows discrimination between living and dead cells. In this paper we show for the first time using flow cytometry that there is a Ca^{2+} increase during capacitation in human sperm. Our measurements provided fluorescence values for each individual cell, so that the distribution of these values in the population can be clearly observed, in turn enabling elimination of the signal from dead cells (Figure 4).

Fluorescence-based imaging assays on intact living cells complements the previous three techniques because it adds spatial resolution to the measurements. High temporal and/or spatial resolution during imaging may also be obtained, according to the available instrumentation capabilities (i.e. camera sensitivity, illumination source, objective numerical aperture, etc.). However, given that only a few cells are studied at once, experiments must be repeated several times in order to draw reliable conclusions, making the analysis time-consuming.

In this paper we used simple experimental protocols in order to illustrate each one of the four techniques. However, these approaches may be extended to study processes in much greater depth. There is a large number of fluorescent dyes that can be targeted to specific compartments of the cell (cytosol, mitochondria, ER, endosomes, etc.); there are ratiometric dyes (double emission and/or excitation wavelengths) that allow quantitative determination of the parameter being measured (calibration is required); finally, more than one dye may be used at once to simultaneously measure, for example, $[Ca^{2+}]_i$ and $[pH]_i$ changes. Additionally, these techniques may be combined with pharmacological tools and/or carefully designed protocols (varying extracellular Ca^{2+} concentrations, pH values, temperatures, or any other factor that may affect the response) to obtain information that may be used to elucidate in detail the process under study.

Disclosures

We have nothing to disclose.

Acknowledgments

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Figure Legends:

Figure 1. Schematic diagram of the experimental protocol for sperm sample preparation by the swim-up method. The major steps for separation of motile sperm and for adjustment of their concentration are illustrated. The last incubation step is only performed when capacitation is required.

Figure 2. Progesterone causes an increase in $[Ca^{2+}]_i$ in human sperm populations. (A) Representative fluorescence traces vs. time showing $[Ca^{2+}]_i$ changes caused by the addition of 4 μ M progesterone (Pg, red trace), or HSM with 0.01% DMSO (Pg vehicle) as negative control (Ctl, blue trace). As a positive control, the change induced by 10 μ M ionomycin (Iono) is shown for each trace. Fluorescence values were acquired at a frequency of 0.5 Hz during 120 s. (B) Bars represent the average ΔF from each condition \pm SE, $n = 3$ (* $p < 0.001$ compared with the control).

Figure 3. Progesterone and ionomycin induce a fast $[Ca^{2+}]_i$ increase in human sperm populations. $[Ca^{2+}]_i$ responses were recorded by sampling every 10 ms (A.F.U.= arbitrary fluorescence units). (A) Raw traces for the control (mixing cells with HSM, green trace) and for signals induced with 10 μ M progesterone (red trace) or with 10 μ M ionomycin (blue trace). (B) Corrected traces for the signals induced with 10 μ M progesterone (red trace) or with 10 μ M ionomycin (blue trace), obtained by subtracting the control signal (green trace in panel A) from their corresponding raw signals (red and blue traces in panel A, respectively).. Progesterone induces a transient response, with a maximum fluorescence value occurring 2.7 s after addition of the inductor. On the other hand, ionomycin generates a rapid and sustained response throughout time (50 s). The inset in panel B shows an expanded view of the first 500 ms for each response. Note that both ionomycin- and progesterone-induced $[Ca^{2+}]_i$ increases start immediately after mixing the cells with the inductor. Each trace represents the average of three recordings. All traces were normalized against their minimum fluorescence values and superimposed on each other.

Figure 4. $[Ca^{2+}]_i$ rises during human sperm capacitation. Capacitated (blue plots) and non-capacitated (red plots) sperm cells were subjected to flow cytometry analysis. (A) Forward (FSC) and side scatter (SSC) plots; the selected gate is indicated with the blue line and only the cells within that area were used for further analysis. Fluorescence histograms in the FITC channel from unstained (autofluorescence) (B) and Fluo-3 AM stained (positive for FITC) (D) spermatozoa. Fluorescence histograms in the PI channel from unstained (autofluorescence) (C) and dead (positive for PI) (E) cells. Two-dimensional fluorescence (Fluo-3 AM and PI) dot plot for unstained (F) and double stained (Fluo-3 AM and PI) spermatozoa (G), the percentage of cells recorded in each quadrant is indicated by the red number. These results are representative of one experiment ($n = 5$).

Figure 5. Progesterone causes an increase in $[Ca^{2+}]_i$ in human sperm cells. (A) Representative pseudocolor images of human sperm cells visualized at the beginning (control), and after progesterone (Pg, 50 s), ionomycin (Iono, 320 s) and $MnCl_2$ (390 s) additions. (B) Representative normalized fluorescence traces of five individual cells from (A).

Figure 1

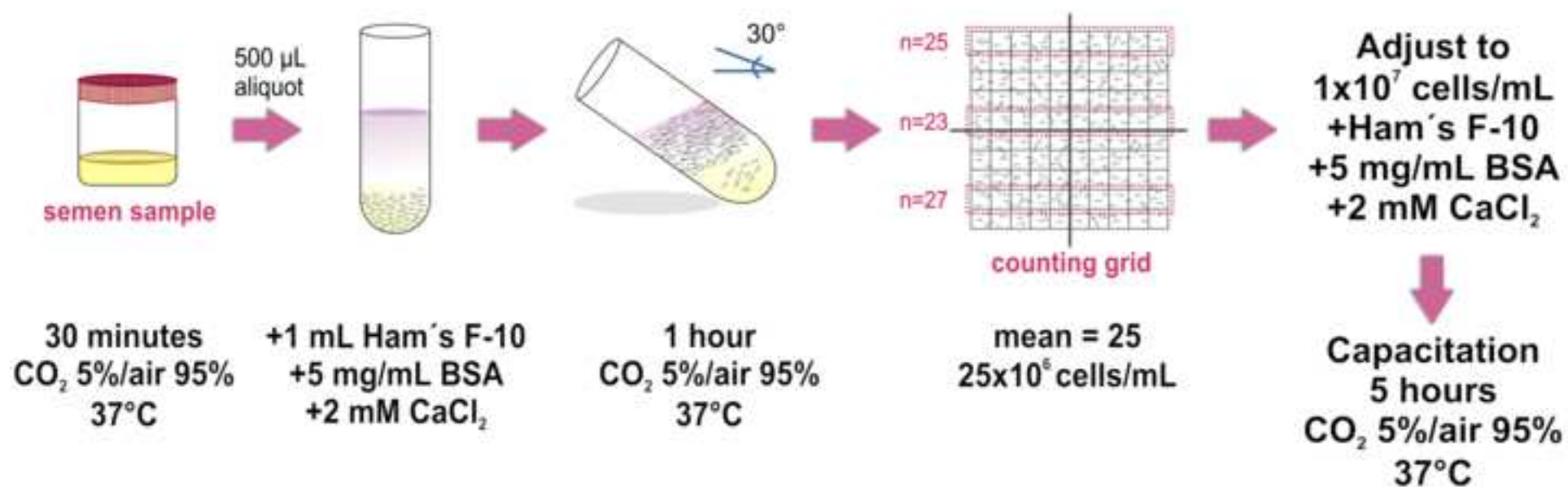
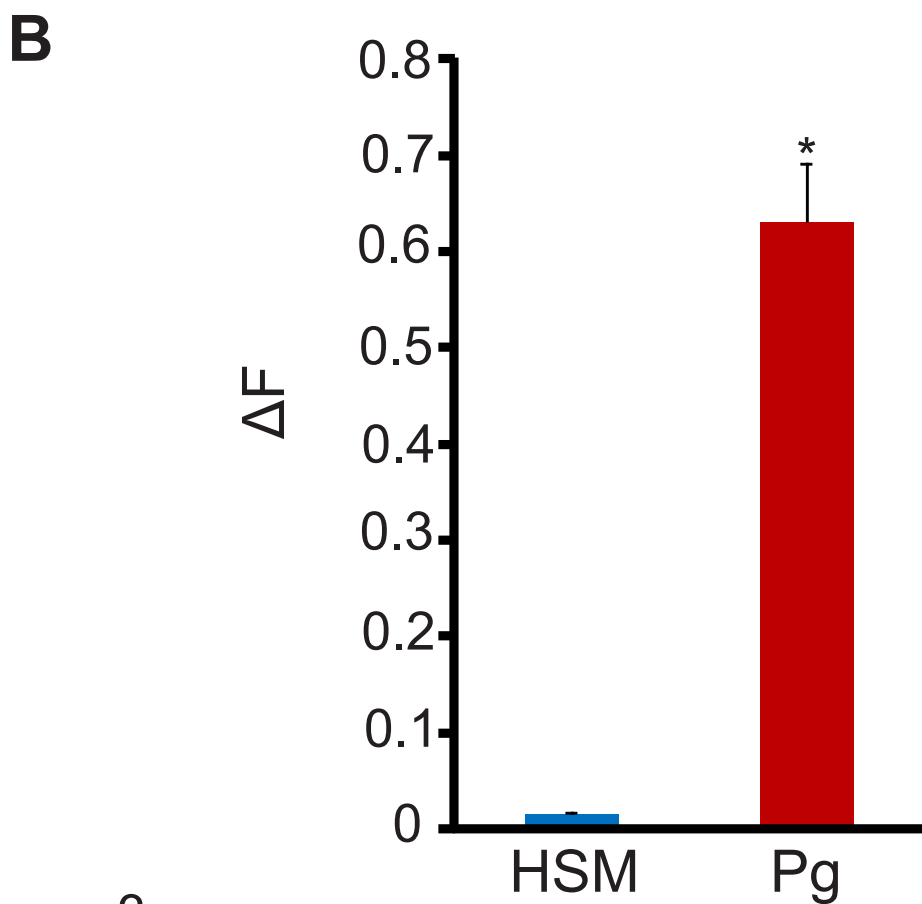
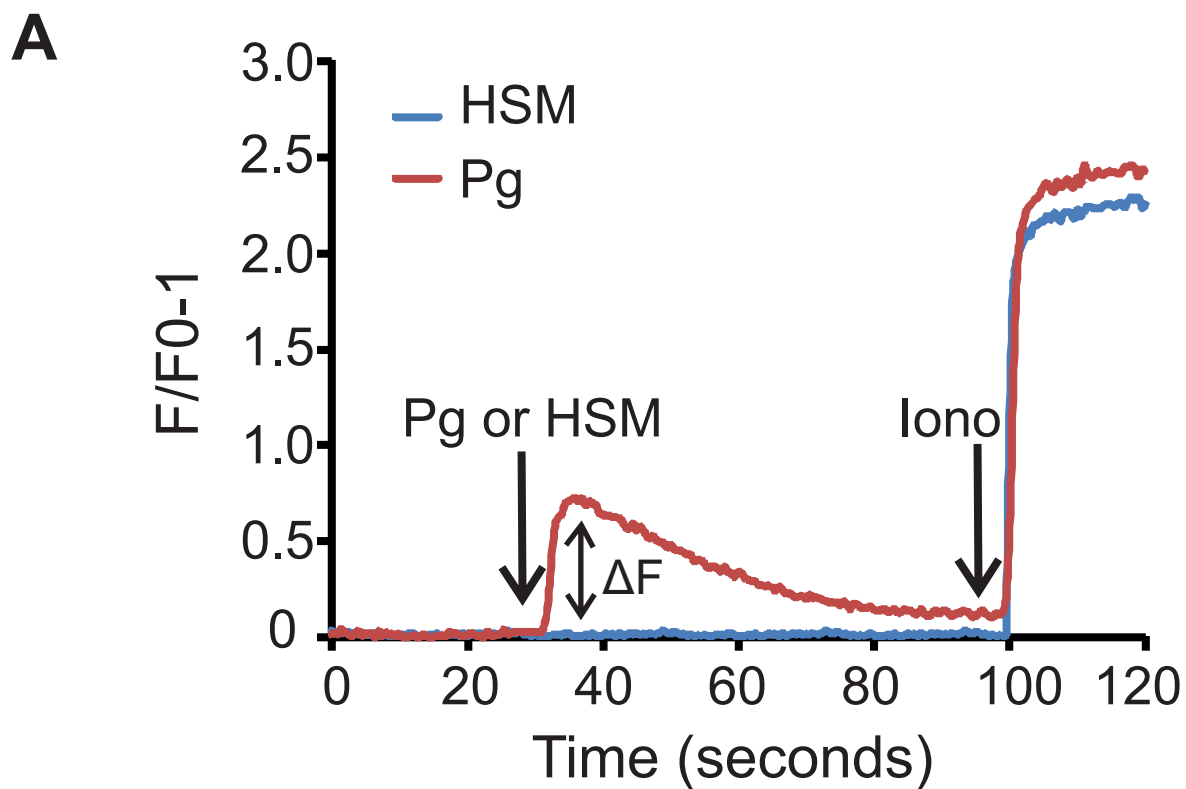


Figure 2



n=3

Figure 3

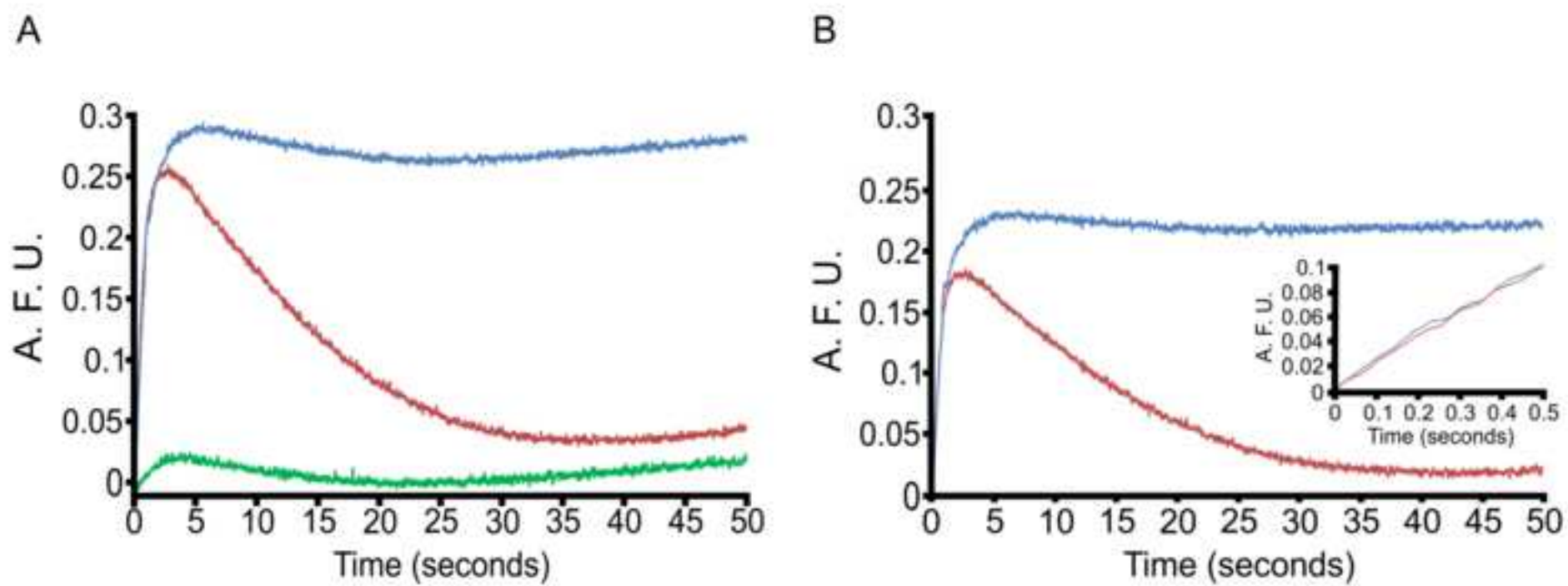
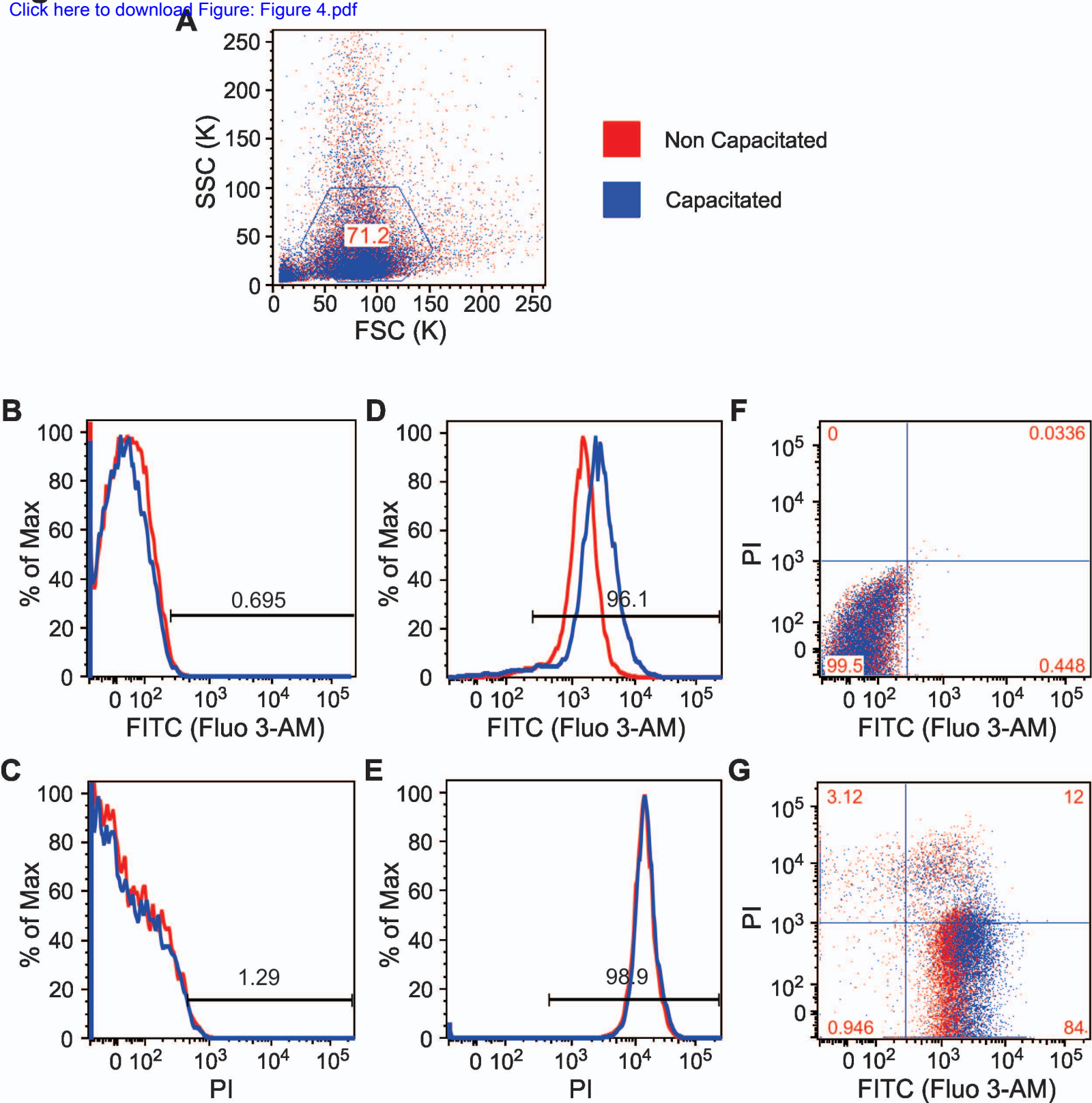
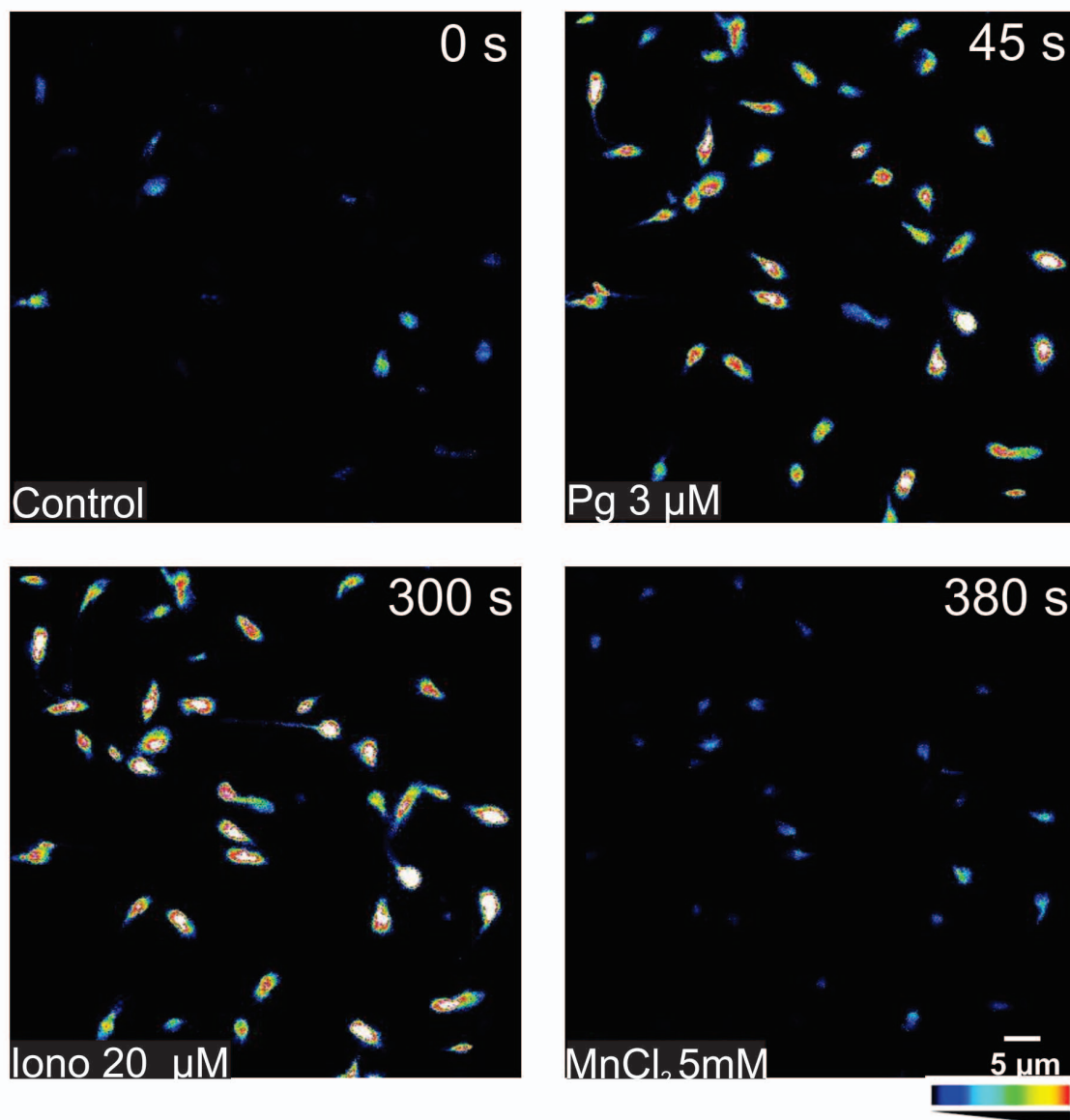


Figure 4[Click here to download Figure: Figure 4.pdf](#)

A



B

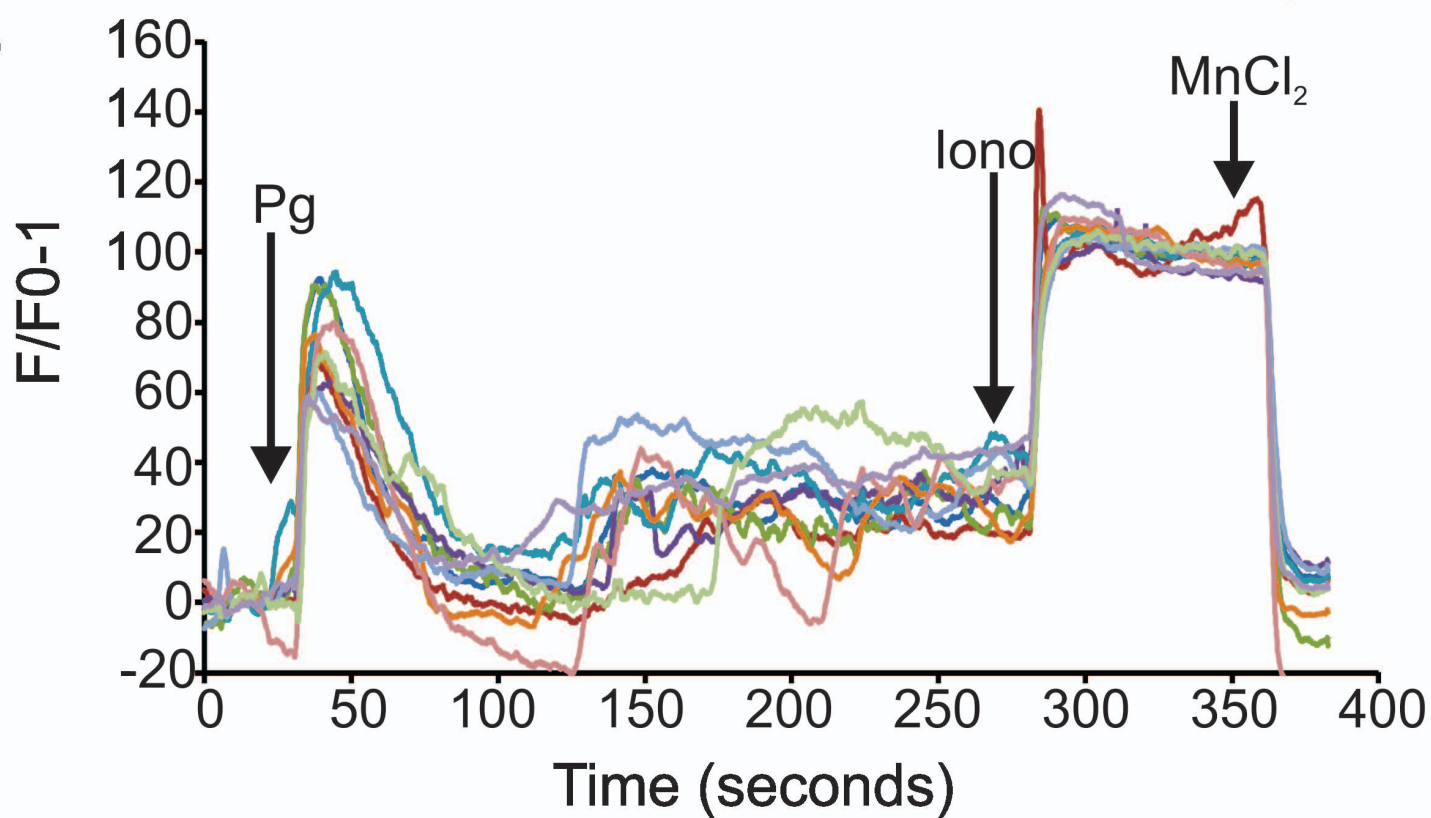


Table 1. Description of samples for a flow cytometer experiment.

Tube	Condition	Treatment	Values to be Measured	Comments	Graph
1	Control (Non-capacitated cells)	Unstained	a) FSC ¹ and SSC ² b) FITC ³ fluorescence c) PI ⁴ fluorescence	a) To determine cell size and granularity and create gate b) FITC ³ autofluorescence c) PI ⁴ autofluorescence	a) Dot plot A, Red b) Histogram B, Red c) Histogram C, Red
2	Control (Capacitated cells)	Unstained	a) FSC ¹ and SSC ² b) FITC ³ fluorescence c) PI ⁴ fluorescence	a) To determine cells size and granularity and create gate b) FITC ³ autofluorescence c) PI ⁴ autofluorescence	a) Dot plot A, Blue b) Histogram B, Blue c) Histogram C, Blue
3	Positive for FITC ³ (Non-capacitated cells)	Label with FITC	FITC ³ fluorescence	To determine the fluorescence in the sample	Histogram D, Red
4	Positive for FITC ³ (Capacitated cells)	Label with FITC	FITC ³ fluorescence	To determine the fluorescence in the sample	Histogram D, Blue
5	Dead cells, positive for PI ⁴ (Non-capacitated cells)	Label with PI	PI ⁴ fluorescence	To determine fluorescence of dead cells	Histogram E, Red
6	Dead cells, positive for PI ⁴ (Capacitated cells)	Label with PI	PI ⁴ fluorescence	To determine fluorescence of dead cells	Histogram E, Blue
7	Control (Non-capacitated cells)	Unstained	FITC ³ and PI ⁴ fluorescence	To determine autofluorescence in FITC ³ and PI ⁴ channels	Dotplot F, Red
8	Control (Capacitated cells)	Unstained	FITC ³ and PI ⁴ fluorescence	To determine autofluorescence in FITC ³ and PI ⁴ channels	Dotplot F, Blue
9	Positive for FITC ³ and PI ⁴ (Non-capacitated cells)	Label with FITC ³ and PI ⁴	FITC ³ and PI ⁴ fluorescence	To determine the fluorescence values in both channels in the experimental condition	Dotplot G, Red
10	Positive for FITC ³ and PI ⁴ (Capacitated cells)	Label with FITC ³ and PI ⁴	FITC ³ and PI ⁴ fluorescence	To determine the fluorescence values in both channels in the experimental condition	Dotplot G, Blue

¹FSC: forward scatter; ²SSC: side scatter; ³FITC: Fluorescein isothiocyanate; ⁴PI: Propidium Iodide

Table 2. Preparation of solutions for an imaging experiment.

Compound	Volume of stock solution to be added	Stock concentration	Volume present in chamber at the time of addition	Final concentration in chamber
Progesterone	100 µL	9 µM	200 µL	3 µM
Ionomycin	100 µL	80 µM	300 µL	20 µM
MnCl ₂	100 µL	25 mM	400 µL	5 mM

Name of Reagent/Material	Company	Catalog Number
Ham's F-10	Sigma-Aldrich	N-6013
Bovine Serum Albumin	Sigma-Aldrich	A-7906
Calcium Chloride Dihydrate approx. 99%	Sigma-Aldrich	C-3881
Makler Counting Chamber	SEFI Medical Instruments LTD	SEF-MAKL
Fluo-3 AM	Invitrogen	F-1242
Ionomycin	Alomone	I-700
Progesterone	Sigma-Aldrich	P0130
Sodium chloride	Sigma-Aldrich	S-9888
Potassium chloride	Sigma-Aldrich	P-3911
Sodium bicarbonate	JT Baker	3506
Magnesium chloride	Sigma-Aldrich	M-2670
Calcium chloride anhydrous	Sigma-Aldrich	C-1016
HEPES	Sigma-Aldrich	H-3125
D-Glucose	JT Baker	1906-01
Sodium pyruvate	Sigma-Aldrich	P-2256
Sodium L-lactate (aprox. 99%)	Sigma-Aldrich	L- 7022
Propidium Iodide	Invitrogen	L-7011
Triton X-100 (t-Octylphenoxypolyethoxyethanol)	Sigma- Aldrich	X-100
Round coverslip	VWR	48380 080
Poly-L-lysine solution	Sigma-Aldrich	P8920
Manganese chloride	Sigma-Aldrich	M-3634
Attotfluor® Cell Chamber, for microscopy	Life technologies	A-7816
Dimethyl Sulphoxide	Sigma-Aldrich	D2650

Comments
20 vials/50 ug each
Reagents for human sperm medium (HSM)
Component B
2.4 mM solution in water
25mm diameter
5x5mL

Equipment
[Click here to download Table of Reagents/ Materials Used: Equipment Table.xlsx](#)

Name of Equipment	Company	Catalog Number
SLM Aminco Spectrofluorometer	SLM instruments	JD-490
Blue LED	Lumileds Lighting LLC, San José, CA, USA	LXHL-LB3C
Bio-Kine32 Analysis Software	BioLogic Science Instruments	N/A
SFM-20 Stopped Flow Mixer	BioLogic Science Instruments	054-00/21
FACSCanto™ II flow cytometer	Becton Dickinson	338960
FlowJo software	Tree Star	9.3.3
iXon EMCCD camera	Andor Technology, Belfast, Northern Ireland	DV887ECS-BV-500
Imaging Software	Andor	IQ
Imaging Software	National Institutes of Health	Image J
Inverted Microscope	Nikon, Melville, NY, USA	Nikon Diaphot 300
Nikon PlanApo 60× objective	Nikon, Melville, NY, USA	60XWI

Comments (optional)
Operated by OLIS Global Works softwear
With 465-505 nm band-pass filter (Chroma Technology Corp.)
Equipment with software included
6-colors, blue/red lasers
Public domain software

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Summary

The manuscript entitled "Measuring intracellular Ca^{2+} changes in human sperm using four techniques: fluorometry, stopped flow fluorometry, flow cytometry and single cell imaging" by Mata-Martinez and co-authors compares different fluorescence-based techniques to measure Ca^{2+} -changes in human sperm. The manuscript is well written and gives an overview of the most common methods of measurement of Ca^{2+} sperm.

However, I have some comments/concerns:

Major Concerns

1) The authors state in the introduction ".....only capacitated sperm can undergo the acrosome reaction....". Do the authors mean an acrosome reaction evoked by agonists or a physiological acrosomal exocytosis?

We thank the reviewer for this comment as our statement is indeed misleading. We have modified the statement in the introduction to read as follows: "...It is generally accepted that only a subpopulation of capacitated sperm undergo the acrosome reaction when exposed to physiological inductors..."

2) Also in the introduction it reads "...We monitored fluorescence changes as a function of time with three...". I would suggest writing "In vitro we monitored fluorescence....."

We agree that it is worthwhile specifying this, and we have modified the text as suggested by the reviewer.

3) Some parts of the manuscripts are written more in a lab-book style. For instance under point 3.2 the authors write "Start the experiment using the Olis software, and proceed to acquire..."; under point 6.9 "Image analysis may be performed offline using IQ Software..."; under point 5.5 "At the end export all data.". Does that mean you need to buy special software? What does that software do? Export the data to what format/other program?

The statement in the discussion "....the required instrumentation is readily available in most laboratories..." is not compatible with the use of specific software that has to be bought.

The text was written in lab-book style according to JOVE's preferred format, and the editor has requested that that the format remains as is. Regarding the software, every instrument is sold along with the software required for its operation and for data acquisition. Accordingly, we provided the names of the software required to operate and acquire data when using our specific equipment. And even though data analysis may also be performed using the same software, this is often times impractical as it prevents access to the instrument for experimentation by other users (unless additional software access keys are purchased so that it may be used simultaneously on separate computers). As an

alternative, data analysis may be performed using available freeware. We have slightly modified the text to address the reviewer's questions, and to provide information about additional alternative freeware that may be used for data analysis.

3.2. *Start the experiment using the equipment's software (Olis software in this case) and proceed to acquire fluorescence values at a frequency of 0.5 Hz during 600 s.*

6.9 *Perform image analysis online using the equipment's software, or offline using either IQ Software or ImageJ freeware).*

5.5 *Run all experimental tubes (in this case, tubes 7-10). At the end, export all data to the software available for analysis (see Step 5.6).*

5.6 *Analyze the results of each experiment using the equipment's software, the commercially available FlowJo software or Cytobank freeware (<http://www.cytobank.org/>).*

We believe we can keep the statement "....the required instrumentation is readily available in most laboratories...", given that (a) having access to any particular piece of equipment implies the availability of its corresponding software for operation and data acquisition, and (b) we are now providing freeware alternatives for data analysis.

Minor Concerns

4) Under "Representative Results; Technique #1" the authors write "Progesterone is one of the known physiological AR inducers...". This statement is - at the least -controversial.

We appreciate the reviewer's correction, and we have omitted the word 'physiological'.

5) Figure 2: Is Ctl the same as HSM? Why did the authors use different abbreviations?

This was an oversight on our part, and we have unified the nomenclature; both panels are now labeled HSM.

Reviewer #2

Summary

This is a well-written and timely account of various techniques for measuring $[Ca^{2+}]_i$ signals in sperm and how they are applied. This is an area where there is a lot of research activity at the moment and this is a very useful addition.

Minor Concerns

1. The paper includes use of stopped-flow analysis to visualize rapid kinetics of progesterone

activated signals. The authors mention that they used the minimum flow rate to minimize cell damage. The authors explain that a signal is obtained in the control experiment (no agonist) due to the effect of mixing and that this is subtracted from the experimental trace. This is good but it would be useful to see traces showing what occurs in the control experiment and how it depends on flow rate. Sperm are responsive to shear, which increases $[Ca^{2+}]_i$, and its therefore at least possible that the stopped flow technique, in addition to the effects seen in the control, also affects the response to progesterone?

The reviewer is absolutely right; sperm cells most likely possess pressure-sensitive Ca^{2+} channels, and this is precisely the reason for subtracting the response obtained solely from mixing. But we agree that it is worthwhile including the results for the control experiment, and therefore we have modified Figure 3 in order to include both raw and subtracted traces.

2. The imaging technique described here uses a static bath with the stimulus being applied using a micropipette. This clearly works well as the data in fig 5 shows but it does have some drawbacks. It is worth mentioning that use of a perfusion chamber is an alternative that allows stimuli to be removed as well as added and also gives greater precision in drug application as there is no dependence on diffusion. Perfusion also has its drawbacks such as effects of fluid shear and problems with temperature maintenance.

This is an excellent suggestion and we have incorporated this alternative by adding the following statement:

Alternatively, compounds may be added using a perfusion chamber which offers the advantages of enabling stimulus removal, and the ability to uniformly bathe the cells with the compound. At the same time, it does have the disadvantages of requiring larger quantities of solution, and of making temperature control more problematic.

Please note that even though Reviewer 1 states part of the protocol being *written in a laboratory notebook style* is a concern, this is the preferred format and it should not be changed.