**Reviewer #1**   
*Summary*   
The manuscript entitled "Measuring intracellular Ca2+ 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 Ca2+-changes in human sperm. The manuscript is well written and gives an overview of the most common methods of measurement of Ca2+ 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/ 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 [Ca2+]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 [Ca2+]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 Ca2+ 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.