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# Fluorescence Technique Description for Measuring $Ca^{2+}$ in Mice Sperm

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## Abstract

**Recently, fluorescence technique becomes very useful. It can allow for addressing a fundamental problem of cellular mechanism besides characterizing the species inside the cell to facilitate the diagnostic and prognostic value. Manipulation with fluorescent dyes provides many possibilities for their use as tags, probes, and sensors. These types can be intrinsic or extrinsic to the cell. They can become not only silent observers, but also participants, modulators or disruptors of specific activities outline the biological functions can be successfully studied quantitatively and qualitatively with fluorescence techniques.**

## Keywords

**$Ca^{2+}$ Imaging, Sperm Mice, $Ca^{2+}$ Channel, Fluoresce Microscopy**

## 1. Introduction

Sperms are specialized cells, have a vital role in fertilization through initiation of several events, induce $Ca^{2+}$ signaling, opening plasma membrane $Ca^{2+}$ channels and allow the entry of $Ca^{2+}$ which is required for the sperm to reach the oocyte and induce acrosome reaction. The essential requirements to achieve fertilization such as the hyperactivation, chemotaxis and acrosome reaction are $Ca^{2+}$ signaling to arrange all these activities [1]. Otherwise, the sperms have the same $Ca^{2+}$ signaling in the other cells, as like neurons. For example, the releasing of $Ca^{2+}$ from intracellular stores or entry the extracellular $Ca^{2+}$ inside both sperm and neurons can lead to increase intracellular $Ca^{2+}$ level inside these cells [2] [3]. The male infertility related to impairment of $Ca^{2+}$ signaling [2]

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[4] [5]. The $Ca^{2+}$ channels found in the plasma membrane of sperm cells play an important role for $Ca^{2+}$ signaling to improve sperm cell activities [6]. Most studies have confirmed that the changes in $Ca^{2+}$ signaling contribute to the regulation of many different functions of mammalian sperm cells [1] [7]-[10]. Spermatozoa produce $Ca^{2+}$ signals in response to extracellular changes, for instance ZP induced acrosome reaction, chemotaxis in response to low dose progesterone [1] [6]. During capacitation, hyperactivation and the acrosome reaction there is evidence for increasing $Ca^{2+}$ concentration in several mammalian species including human [11] [12]. Sperm have many channels on cell membrane provoke $Ca^{2+}$ influxes and internal release of $Ca^{2+}$ through these channels. Ion channels have been playing several important roles in sperm physiology that assist a means of linkage between the sperm and its surrounding. They play essential roles in the arranging of sperm motility, chemotaxis and the acrosome reaction [13]. $K^+$ conductance activated by alkalinization termed KSper, showed to be a primary $K^+$ conductance in mouse spermatozoa [14]. However, it has suggested that hyperpolarization is resulting from alkalinization-induced KSper activation. This work is dealing with the simultaneous activation of the pH-sensitive $Ca^{2+}$ selective CatSper channel to activate $Ca^{2+}$ entry that is essential to induce of the hyperactivation of sperms [14]. KSper activation is proposed to play a critical role in the some of the physiological and biochemical changes assembling called capacitation. That has an essential role to evoke sperm's ability for fertilization [15] [16]. $NH_4Cl$ is a weak base has an essential role in sperm functions through induce alkalinization of a sperm medium and triggered sperm capacitation through elevating internal pH and induce $Ca^{2+}$. Otherwise, fluorometric recordings of sperm loaded with BCECF-AM or fluo3-AM showed elevations of intracellular pH and $Ca^{2+}$ [17], as well as a single-cell image analysis, showed $Ca^{2+}$ elevation levels in the flagellum in response to $NH_4Cl$. In addition, other researchers were reported that $NH_4Cl$ simulate depolarization and induce $pH_i$ alkalinization with an increase in $[Ca^{2+}]_i$ that depended on the amount of added $NH_4Cl$, furthermore if an additional stimulation occurred, calcium release from internal stores [18]. Otherwise, an elevation in intracellular pH and $Ca^{2+}$ of sperm regulate sperm motility, chemotaxis, capacitation, acrosome reaction, and play a vital role in the ability of the sperm cell to reach and fertilize oocyte [19]. Internal pH ($pH_i$) controlling a wide series of physiological processes offers a means of consolidating different cellular functions with metabolic and activity states [20]. In sperm, changes in $pH_i$ to alkaline, the internal pH ($pH_i$) play a vital role in controlling physiological process related to three physiological changed to achieve fertilization, first, $pH_i$ elevation initiate and modulate flagellar motility in non-mammalian sperm and, possibly, in mammalian sperm [21]. Second, the initial of mammalian sperm maturation and fertilization occurs in the female reproductive tract via the process called capacitation and its depending on $pH_i$ elevation [22] [23], finally, sperms of different animal species need achieve acrosome reaction (AR), thus a $Ca^{2+}$ dependent secretory event, prior to fertilization [12], activated $K^+$ conductance has been activated through alkalinization and this conductance termed KSper, seems to be the primary $K^+$ conductance in mouse's sperms [14]. The key element in $Ca^{2+}$ signaling pathway is pH and chemotaxis which control $Ca^{2+}$ dependent responses essential for fertilization in marine invertebrate and mammalian sperm, chemotaxis in invertebrate sperm include such a response, whereby egg peptides induce a pH-mediated $Ca^{2+}$ entry that improve flagellar beating to drive sperm toward the oocyte and produce acrosome reaction [24] [25]. In addition $pH_i$ alkalinization mostly act on $Ca^{2+}$ signaling pathway through evoke a rapid elevation of $Ca^{2+}$ concentration in sperm flagellum for induce hyperactivation [17].

We used fluorescence technique to measure the qualitative level of calcium in sperm through loaded with fluorescent, which is a particular $Ca^{2+}$-sensitive dye, used for measurement a spatial and temporal aspects of $Ca^{2+}$ signaling in living cells. In this study, we determined the method used in our laboratories for loading suspensions of mice's sperm with a form $Ca^{2+}$-loading dyes fluo4-Am then measuring the fluorescence intensity during application of the stimulus.

Fluo4-AM dye is adding to a sperms suspension and 30 minutes is a suitable time for this dye to enter the sperm cytoplasm. A visible wavelength dyes to minimize photo-damage to the sperm and prevent photobleaching during an imaging period. During the loading period, sperms are put in a bottom an imaging chamber coverslip coated of Cell-Tak washing sperm in Hanks medium at pH 7.4 at the end of loading duration, through multiple plastic tubes connecting to a manual perfusion apparatus. The stimuli and Hanks solution are adding to the perfusion header, then prefused contentiously through separated plastic tube to the imaging chamber recorded by time-lapse acquisition for pre-treatment phase (before stimulus application) and at stimulus application. Then recording video and took images of $Ca^{2+}$ changes. At the end of the experiment, the images imported into image J software to analyze manually, select the area of interest by draw a line around whole sperm, including three regions (head, midpiece and tail). Data are normalized by using sigma plot statistic program and plot a graph

showing the $Ca^{2+}$ response represented as $\Delta F/F_0$ (%).

The aim of this case study have done it in our lab, is to describe the details of fluorescent technique is to understand the molecular mechanism of sperm mice specially calcium changes, avoid the disadvantage and mistakes help some beginning researcher who doing this technique for first time. However in current studies some authors showed the section of material method in their published papers in briefly without details. So that, we depended on this experiment on reviewing our materials method according to reference [26] [27] for preparing HS medium and [28] for imaging system and $\Delta F/F_0$ (%) as describes in [29], unless otherwise details is our current study as describe later.

## 2. Requirements for $Ca^{2+}$ Imaging of Mice Sperm

Chemicals and reagents are purchased from Sigma-Aldrich Co. (St. Louis, MO) with the following exceptions unless otherwise stated. Fluo4-AM and pluronic F-127 have obtained from Molecular Probes (Invitrogen, Eugene, OR). Cell-Tak was from BD Biosciences (Bedford, MA).

### 2.1. Supplemented Hanks Solution Medium Preparation Containing in (Mm)

135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 HEPES, 10 Glucose, 10 Lactic Acid, 1 Na pyrovate, adjusted to pH 7.4 with NaOH according to reference [26] [27].

### 2.2. Cell-Tak Preparation

10 µl Cell-Tak, 285 µl Sodium Bicarbonate, and 5 µl 1N NaOH (added immediately before coating) to make 300 µl Cell-Tak solution. The BD Cell-Tak protein deposited on a glass or plastic surface by mechanical spreading. Using a handy tool, such as a glass rod or micropipette tip, microliter volumes of BD Cell-Tak in 5% acetic acid can be spread in a thin liquid film. As the acetic acid evaporates, a coating of BD Cell-Tak is left behind.

### 2.3. Preparation of Dye Stock

Fluo4-AM should be diluted by adding 45.5 µl DMSO to a one bottle of Fluo4-Am to get 1 Mm of it. In addition, then take 1 ml HS add to 10 µl pluronic F-127 following by adding 4 µl Fluo4-AM in the dark, after that the loading dye should be kept in freezing.

### 2.4. Animals

Animals were used are performed Wild-type (WT) followed the guidelines approved by AAALAC Intl (Center for Animal Care) of Wuhan University.

### 2.5. Sperm Preparation for $Ca^{2+}$ Imaging

Adult male mice have used for sperm collection; ages ranged from 3- to 6-month olds. Mice were killed by $CO_2$ asphyxiation, followed by cervical dislocation. Excised the caudal epididymis and rinse with HS medium, sperms were released from three small incisions at 37.8˚C and 5% $CO_2$ & wash in HS with 4 mg/ml BSA. Transfer into 1.5 ml HS with 4 mg/ml BSA and cut into three pieces. Let sperms diffuse for 15 min at 37˚C. Transfer the sperms into 7 ml HS and spin down for 5 min at 400 g (RT). Wash one more time with 7 ml HS; Resuspend in 1 ml HS; Adjust to $1 \times 10^7$/ml.

### 2.6. Coated of Coverslip with Cell-Tak

Coverslip coated with Cell-Tak, Each one needs a ~2 µl immediately before use. The chamber placed in the incubator, Cell-Tak coated coverslip side down slightly smears. Dry ~10 minuets. Sperms then fixed on the area coated with Cell-Tak. After that, washing chamber with distilled water.

### 2.7. Fluorescent Labeling

100 µl fluo4-AM dye added to 200 µl HS sperm suspension and add. Incubate in dark at room temperature for

30 min. After that, it followed by twice washing with HS.

## 2.8. Chamber Mounted on the Microscope

Chamber then mounted on the microscope connected to the perfusion apparatus and perfuse by multiple plastic tubes. A roller pump feeds HS into the chamber. The preparation is left in the dark for at least 10 min while a perfusion of the chamber removes dead cells and extracellular dye. Temperature stability is significantly important because small fluctuations may not only affect cell $Ca^{2+}$ homeostasis but may also affect Kd of the dye. Experiment has performed at room temperature.

## 2.9. Confocal Machine

Andor Technology (Springvale Business Park, Belfast, UK) with 75 WXenon lamp, was used to generate excitation at 488 nm. Microscope (IX-71; Olympus) used for imaging objective lens is 60×. Emissions 491 nm was filtered through a band-pass filter (HQ540/50; Chroma, Rockingham, VT). Signals collected through a cooled; charge-coupled device camera (CoolSNAP HQ; Roper Scientific, Trenton, NJ) [28] as showed in **Figure 1**.

## 2.10. Imaging Steps

Sperms observed under objective microscope (60 × oil-immersions), selection of an area of imaging is should have fixed, non-motile and survive sperms, but motile even dead sperm should be excluded from imaging. A part of coated area by Cell-Tak coated having sperms selected for imaging should be near the inlet port of plastic tube tip, where HS flow through perfusion apparatus. With considering choosing an area where sperm has an adequate amount of fluorescent intensity, to prevent affects the quality and sincerity of data through signal reception of from adjacent cells. In addition, where sperms have fixed, however, flagellar activity is discernible [29]. The imaging contrast phase, should be saved, after selection the area of imaging, visible light of microscope should be closed, then turn to switch the microscope to fluorescence mode (emission 491 nm) and time of exposure to obtain a clear fluorescence image here we selected time per 5 seconds (**Figure 2**).

1) A negative control duration have determined during recording, manipulation of HS constituents from perfusion header by opening the gate to flow HS through plastic tube, considering it as positively control. The advantage of this method is to make sure that the sperms signals are stable after HS flow to the imaging chamber. Time of addition here should be on record. After a period determined, close the HS gate from the perfusion header and open stimulus gate also here the time of adding and the duration period of each stimulus have determined.

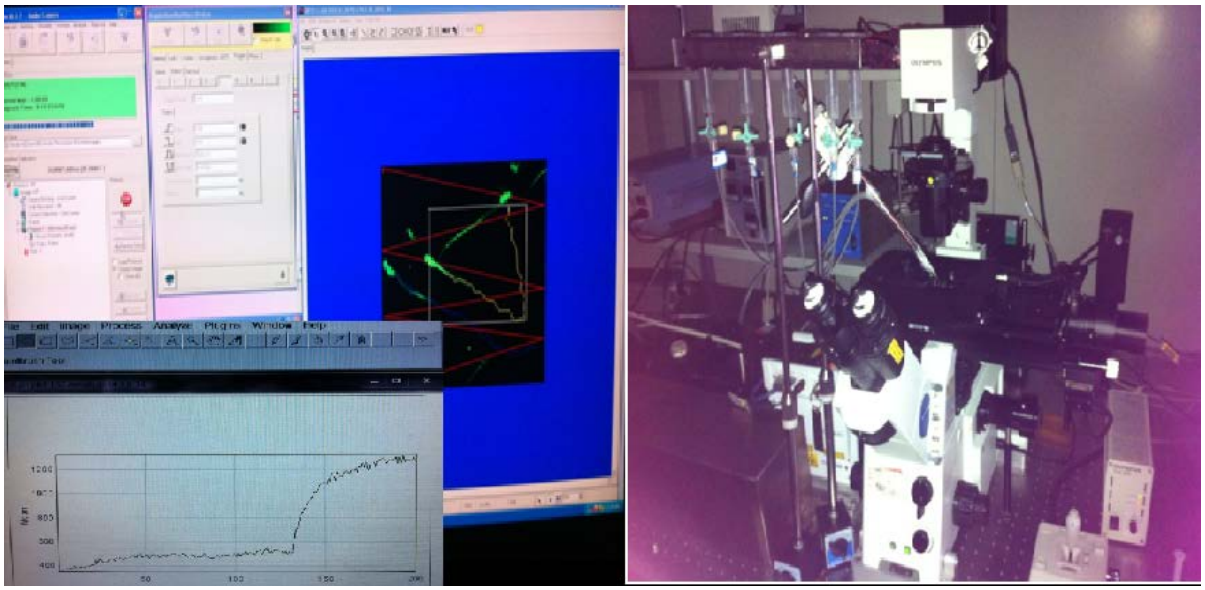

**Figure 1.** Single sperm was imaging by using confocol laser microscope and perfusion apparatus.

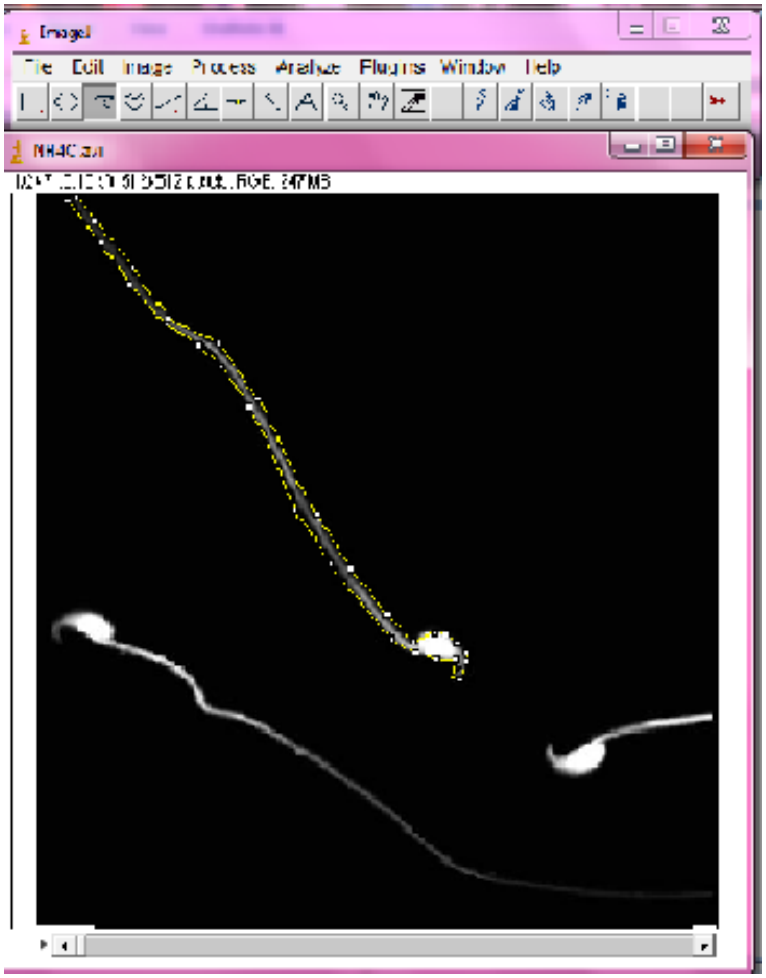


**Figure 2.** Region selected of sperm for analysis, whole sperm was marked by ROIs by using image J software.

2) Images are analyzed using image J software. ROI have drawn around the whole sperm including three regions (head, midpiece, and tail) and also the free area near the sperm selected for background subtraction as showed in **Figure 2**. In addition, this software has automatic subtraction by selected the function (process—background subtract). Sperms were excluded from the analysis if they moved outside the region of interest if the fluorescence faded to zero within the duration of the experiment (assumed to reflect the loss of dye due to cell death). Analysis here done after experiment after saving images and movie to transfer it to another computer have image J software downloaded.

## 3. Data Analysis

Fluorescence intensity series value obtained from video analysis and these data analyzed by imported the raw intensity value got from image J software in a sigma plot analyzing program. Firstly, fluorescence value normalized to the mean value obtained during the control period. $[Ca^{2+}]_i$ changes were represented as $\Delta F{:}F_0$ ratios after background subtraction, where $\Delta F$ was the change in fluorescence signal intensity, and $F_0$ was the baseline calculated as the average of the 10 - 20 frames prior to stimulus application [28]. Raw intensity values were imported into sigma plot, analyzing programs and normalized utilizing the equation as described below in details as showed below:

$$F = \Delta F\left(Fp - F\,\text{rest}\right)/\left(F\,\text{rest} - Fb\right)\times 100\%$$

F = is normalized fluorescence intensity;

$\Delta F$ = change in fluorescence signal intensity calculated as (Fp − $F_0$ rest);

Fp = fluorescence intensity during adding stimulus;

$F_0$ rest = fluorescence intensity at 0 times (before stimulus application) is the mean of 10 - 20 determinations of F obtained during the control period;

Fb = fluorescence intensity after background subs traction = (F-background);

The final normalized fluorescence intensity is represented as $\Delta F/F_0$ (%) as describes in [29].

## 4. Representative Results

**Figure 3** shows pseudo-color images series (warm red colors show high $[Ca^{2+}]_i$ of Fluo4-AM fluorescence response in a "typical" sperm cell at various points in the experiment). The representative images showed the $[Ca^{2+}]_i$ changes evoked by the above stimuli along the flagella of sperms. The changes of the $Ca^{2+}$ signals started from the tail, and then propagated to the head. Time points are 0, 160, 460, 560, 660 and 800 s. 10 mM $NH_4Cl$ perfuse into the imaging chamber at 460 seconds.

After perfuses 10 mM $NH_4Cl$ at time is marked by the blue arrow. Baseline characterized by green arrow shows mean control of fluorescence.

Treated extracellular by HS at $pH_8$ after one minute of the control period, caused a slight increased in $Ca^{2+}$ oscillates represented as $F/F_0$ (%), (N = 3).

## 5. Discussion

Confocal recordings of sperm loaded with or Fluo4-AM, revealed that $NH_4Cl$ evoked elevations of intracellular pH and $Ca^{2+}$, respectively, with the rise in pH occurring more rapidly than that of $Ca^{2+}$. Single-cell image analysis showed increased $Ca^{2+}$ levels in the whole sperm in response to $NH_4Cl$ as showed above in represented results **Figures 3-5**. Recent work has demonstrated that the capacitation of sperms activated by membrane hyperpolarization resulting from Ksper activation under alkaline environment is critical for producing fertilization [30] [31]. The representative images showed the $[Ca^{2+}]_i$ changes evoked by the above stimuli along the flagella of sperm mice. Changes of the $Ca^{2+}$ signals started from the tail, and then propagated to the head.

This technique was permitting kinetics and spontaneous recording to induce $Ca^{2+}$ signaling in mice sperm. Although, responses can be obtained from mice sperms by a single cell imaging at least 1 - 2 sperms. Whose are quite different from human sperms recording more than 200 sperms can be done in the same experiment, but this can illustrate different responses to induce $Ca^{2+}$ signal.

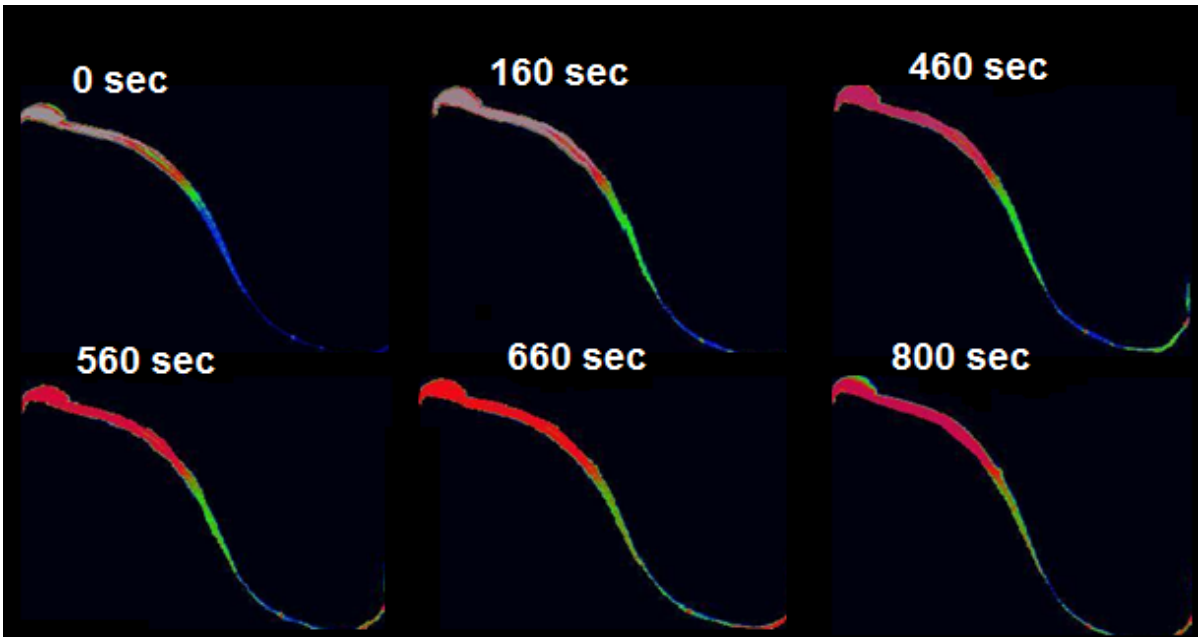


**Figure 3.** $Ca^{2+}$ changes of sperm samples treated by 10 mM $NH_4Cl$.

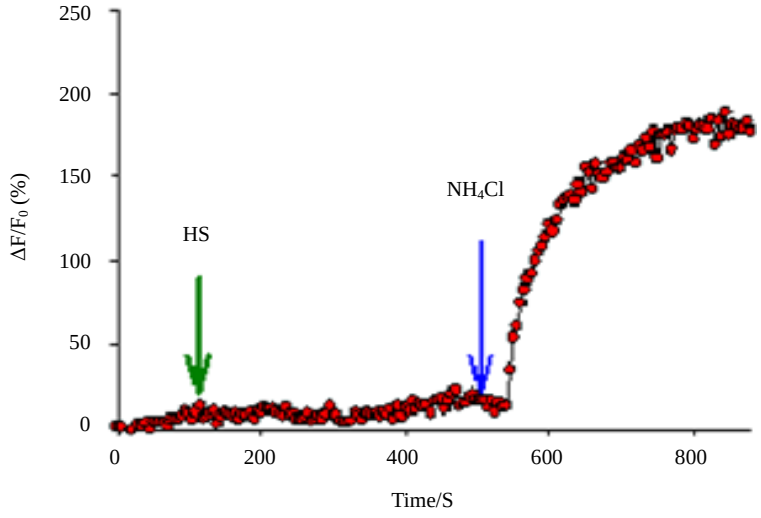


**Figure 4.** Increase in the relative florescent intensity represented as $\Delta F/F_0$ (%).

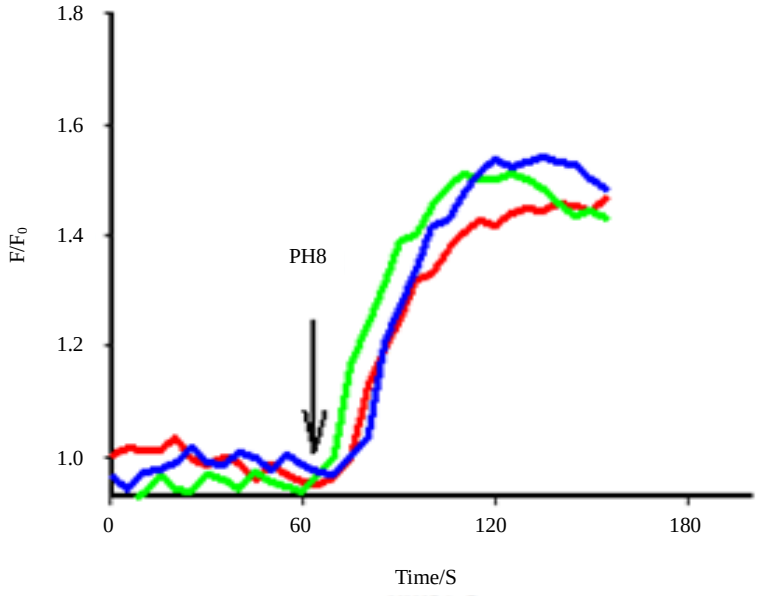


**Figure 5.** Fluorescence-time traces for three individual sperms plotted with different colors lines.

Right florescent loading, sperm survival, and healthy sperm fixation while recording can achieve good result. The weak sperm samples give weak record; weak signals in an addition sperm may well die during recording. As well as the temperature of the experimental environment should be maintained at a degree of room temperature. Both higher and lower temperature in vitro have an adverse effect on sperms survival, fluorescent labeling then responses to stimulus this will subsequently give useless results. Mice sperm has sensitivity to light exposure photo-damage. For this reason took in our mind to use the minimal illumination (both intensity and exposure time), in order to, maximize the possibility of sperm survival and preventing sperm die.

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