Stroboscopic illumination using light-emitting diodes reduces phototoxicity in fluorescence cell imaging

Takuya Nishigaki[1], Chris D. Wood[1], Kogiku Shiba[2], Shoji A. Baba[2], and Alberto Darszon[1]

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Excited fluorophores produce reactive oxygen species that are toxic toward many live cells (phototoxicity) and accelerate bleaching of the fluorophores during the course of extended or repeated measurements (photobleaching). We recently developed an illumination system for fluorescence microscopy using a high power light-emitting diode (LED), which can emit short pulses of light (0.5–2 ms) to excite fluorophores. This system minimizes illumination time, thus reducing phototoxicity and photobleaching artifacts. To demonstrate the usefulness of the new system, we compared images of human sperm loaded with various fluorescent indicators and excited with either a conventional mercury lamp as a continuous excitation light source or the LED as a source of pulsed illumination. We found that sperm motility decreased rapidly and photobleaching was relatively rapid under continuous illumination, whereas under pulsed LED illumination, motility was maintained and photobleaching was much reduced. Therefore, fluorescence microscopy using LED-based pulsed illumination offers significant advantages for long-term live cell imaging, reducing the degree of phototoxicity, and extending the effective lifetime of fluorophores.

INTRODUCTION

Fluorescent imaging of live cells is one of the most important strategies to study real-time cellular responses. Besides conventional small fluorescent indicators, such as fura-2 (1) and fluo-3 (2) to measure Ca^{2+} concentration, there are now many fluorescence resonance energy transfer (FRET)-based fluorescent indicators to measure biological parameters like cyclic nucleotides (3,4) and several other metabolites (5). To capture a clear fluorescent image with a high signal-to-noise ratio, it is important to detect emitted fluorescence light as efficiently as possible utilizing a high numerical aperture objective lens, a wide band-pass filter, and a sensitive charge-coupled device (CCD) camera (6). For further improvement of the fluorescent signal, a commonly used strategy is to increase the intensity of excitation light or the concentration of the fluorophores. A significant side effect of these procedures is that they increase the generation of reactive oxygen species (ROS), such as singlet oxygen (6), an excess of which will damage the cell (phototoxicity) and increase the rate of photobleaching of the fluorophore itself (6). Indeed, fluorescence detection of ROS often suffers from the signal amplification because of self-generation of ROS by the indicator (7).

We recently developed a stroboscopic fluorescence microscopy technique using a high power light-emitting diode (LED) to study the relationship between cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]i$) and flagellar form in swimming sea urchin sperm (8). In our system, a fluorescent Ca^{2+} indicator, in this case fluo-4, was excited by short pulses of light (1 ms) generated by the LED. This enabled us to capture sharp and clear fluorescence images from the rapidly moving flagellum (about 40 Hz in the case of sea urchin sperm). The stroboscopic illumination system is not just useful for capturing "frozen" images of a rapidly beating flagellum, however, as the reduced illumination time should decrease the generation of ROS and offer protection from phototoxicity and photobleaching during live cell imaging. In this study, we use motile human sperm as a model cell system and demonstrate how the LED-based pulsed illumination system has advantages against conventional illumination system for fluorescence imaging of live cells.

MATERIALS AND METHODS

Materials

Semen was obtained from volunteer donors by masturbation after at least 2 days of abstinence. After liquefaction, 1.5 mL Ham's F-10 was applied to 1 mL semen to allow the motile sperm to swim up into the upper layer of the

[1]Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico and [2]Ochanomizu University, Tokyo, Japan

suspension (1 h at 36°C, 5% CO_2). Swim-up sperm were collected and washed twice by centrifugation (750× *g* for 5 min at room temperature) and suspended in the experimental medium: 120 mM NaCl, 4 mM KCl, 15 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM HEPES, 10 mM D-glucose, 1 mM sodium pyruvate, and 5 mg/mL bovine serum albumin (BSA), pH 7.4, by NaOH. Fluo-3 AM, Fura Red AM™, BCECF-AM, and Pluronic® F-127 were from Molecular Probes™ (Invitrogen, Carlsbad, CA, USA). Poly-L-lysine was from Sigma (St. Louis, MO, USA).

Loading of Fluorescent Indicators Into Sperm and Immobilization of Sperm Onto a Coverslip

Human sperm (1–8 × 10^7 cell/mL) were incubated with 2 µM fluo-3 AM, 10 µM Fura Red AM, or 2 µM BCECF AM plus 0.2% (w/v) Pluronic F-127 for 1 h at 36°C. To remove an excess of the fluorescent indicator and Pluronic F-127, sperm were washed by centrifugation (750× *g* for 5 min at room temperature) and resuspended in the same volume of the medium.

A poly-L-lysine-coated (20 µg/mL) round coverslip (25 mm diameter) was mounted in a temperature-controlled chamber (Harvard Apparatus, Holliston, MA, USA), and 50 µL human sperm suspensions were deposited on the coverslip and left for 5 min. Sperm unattached to the coverslip were removed by washing, and the chamber was filled with 250 µL medium. Regions where 50% of sperm are attached to the coverslip only at the head region (with their flagella moving freely) were selected for imaging. After the temperature of the chamber reached equilibrium (around 30°C in the observing area), fluorescence imaging was initiated.

Fluorescence Imaging System

Figure 1 shows a diagram of the imaging equipment used in this study. Epifluorescence images were collected with a Nikon PlanApo 60× (1.4 NA oil immersion; Nikon, Melville, NY, USA) objective using a Chroma filter set (Ex, HQ470/40×; DC, 505DCXRU; Em, HQ510LP; Chroma Technology, Rockingham, VT, USA) mounted on a TE300 Eclipse microscope (Nikon). The LED (Luxeon V Star Lambertian Cyan LED; Lumileds Lighting LLC, San Jose, CA, USA) was attached on a custom-built aluminum holder and mounted in a FlashCube40 assembly (Rapp OptoElectronic GmbH, Hamburg, Germany) connected to rear epifluorescence illumination port of the microscope. The LED was controlled by custom-built stroboscopic power supply that provided 3 A current pulses of 1 ms duration, which was synchronously triggered by the Photometrics® Quantix® 57 CCD camera (Roper Scientific, Tucson, AZ, USA). Images were collected and analyzed with Andor™ iQ software (Andor Technology, Belfast, Northern Ireland). For dual-emission imaging using fluo-3 and Fura Red, we used an Optosplit image splitter (Cairn Research, Kent, UK) with a Chroma filter set (DC, Q595LP; Em1, HQ535/50m fluo-3; Em2, HQ665/65m Fura Red).

For continuous illumination, a conventional 100 W mercury lamp (USH-102DH; Ushio, Tokyo, Japan) was attached to the other end of FlashCube40 together with a *UNIBLITZ*® electroprogrammable shutter system (Vincent Associates, Rochester, NY, USA). A beam splitter (80/20) was inserted into the FlashCube40 to redirect the excitation light from the mercury lamp to the microscope. In some experiments, a neutral density filter was used to reduce the intensity of excitation light of the mercury lamp.

RESULTS

One of the characteristic properties of nearly all sperm cells is their ability to swim by beating their flagella. Once initiated upon spawning or ejaculation, sperm motility may persist from a few minutes to many days, and under laboratory conditions, human sperm remain motile for many hours. We have previously noted that human sperm motility often rapidly declines upon continuous fluorescence illumination, an effect we ascribed to phototoxicity caused by generation of ROS, and for that reason we chose human sperm as a model system to test whether LED-based pulsed illumination could reduce or eliminate such toxic artifacts.

First, we loaded fluo-3, a commonly used fluorescent Ca^{2+} indicator, into human sperm and performed Ca^{2+} imaging in both systems. In the LED pulsed illumination system, most of the sperm maintained their motility, beating

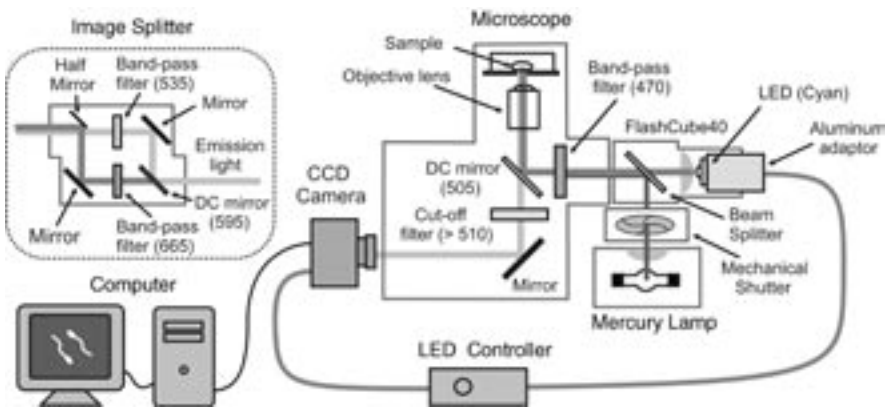

Figure 1. Diagram of the fluorescence imaging system used in this study. Originally, we built this system to perform photolysis of caged compounds using a mercury lamp as a source of UV light during fluorescent imaging as described previously (8). In this study, we used the mercury lamp as a continuous epifluorescence excitation light source. The light-emitting diode (LED) may be used for continuous illumination, however much greater illumination intensities are achieved using a pulsed mode as explained in the Discussion section. The movie images of LED pulsed illumination are available in the supplementary material (Supplementary Movie 1). The image splitter enclosed by dotted line was inserted between the microscope and the charge-coupled device (CCD) camera to perform simultaneous dual fluorescence imaging.

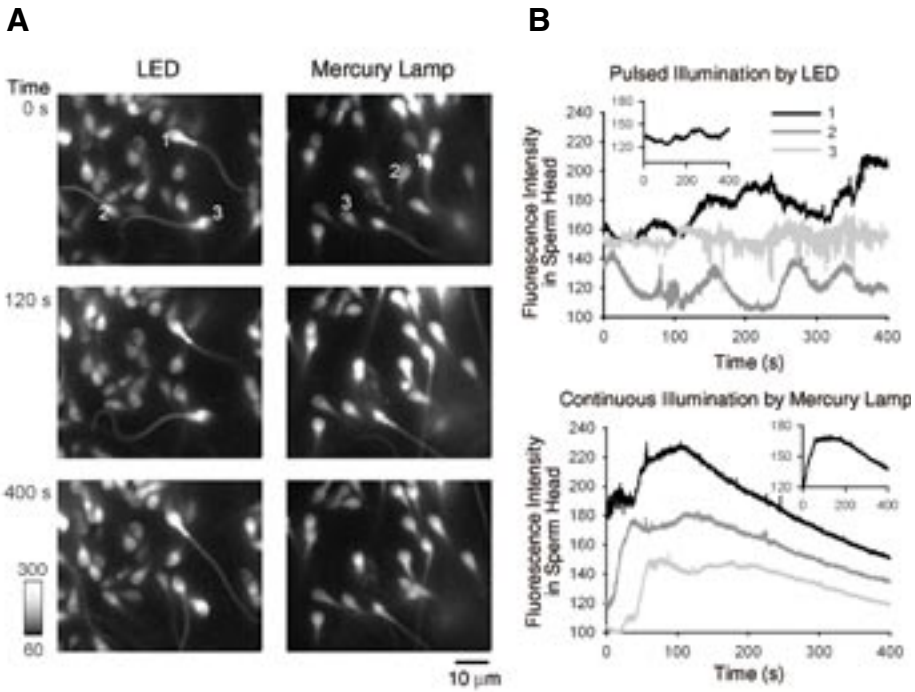

Figure 2. Human sperm Ca^{2+} imaging performed with pulsed illumination of a light-emitting diode (LED) and continuous light from a mercury lamp. Human sperm loaded with fluo-3 were attached on polylysine-coated coverslips, and their fluorescence intensities were measured by two different illumination systems. The exposure time of the charge-coupled device (CCD) camera was fixed at 2 ms in both systems. (A) Fluorescence images of human sperm are expressed using in gray-scale. Most sperm illuminated by pulsed light (LED) remained motile throughout the duration of the experiment, while sperm illuminated by continuous light (mercury lamp) ceased moving around 100 s after the start of illumination. (B) Kinetics of fluorescence intensity in sperm heads using the two different illumination systems. Fluorescence intensities of three individual sperm (head region) are indicated in panel A, with numbers that were plotted for each system. Inset figures indicate the average of three traces in both systems. The results are representative of nine experiments. The movie images are available in the supplementary material (Supplementary Movie 2).

their flagella during the entire imaging period (7 min), and photobleaching was effectively absent (Figure 2 and the supplementary material available online at www.BioTechniques.com). We observed that some sperm undergo spontaneous Ca^{2+} fluctuation, in agreement with an earlier study that recorded similar Ca^{2+} transients (9). In contrast, under continuous illumination with the mercury lamp, sperm motility declined gradually as we had previously observed. Thereafter, the fluorescent intensity of fluo-3 rapidly increased, indicating an increase in $[Ca^{2+}]i$, followed by a more gradual decline in fluorescent signal. Notably, the point at which the fluorescence intensities reached their maximum corresponds to the time when sperm completely stopped moving. The average fluorescence intensities (in the sperm head region) in both systems were comparable (given the identical camera exposure time of 2 ms in each experiment), indicating that the illumination light input from the LED and mercury lamp were of similar intensities. The difference between the two samples is therefore most likely due to the 100-fold increase in overall exposure time to illumination light from the mercury lamp (images/s = 10; duration of individual LED pulse = 1 ms; therefore total exposure to LED illumination/s = 10 ms, compared with full 1000 ms exposure to mercury lamp).

As fluorescence intensity of fluo-3 is highly dependent on the $[Ca^{2+}]i$, then the decrease in fluorescence observed under continuous illumination could be due to either a decrease in $[Ca^{2+}]i$ or photobleaching of fluo-3. To distinguish the two possibilities, Ca^{2+} imaging was performed with Fura Red together with fluo-3. Fura Red can be excited by the same excitation wavelength as fluo-3, but it emits at a much longer wavelength (peak 665 nm), whose intensity decreases upon Ca^{2+} binding in contrast to fluo-3. We used an image splitter to spatially and spectrally resolve the fluorescence emitted by the two dyes simultaneously, as described in the Materials and Methods section and in Figure 1. As shown in Figure 3, both indicators reported the Ca^{2+} fluctuations of human sperm in the LED system without attenuation of sperm motility, in that, when the fluo-3 fluorescence increased, the Fura Red fluorescence decreased. In the continuous illumination system, sperm motility rapidly diminished, while the fluo-3 and Fura Red emissions both indicate a large increase in $[Ca^{2+}]i$ (Figure 4). Following this increase both the fluo-3 and Fura Red signals decrease, suggesting that a photobleaching of both indicators occurred rather than a decrease in $[Ca^{2+}]i$. If a pause of 1 min was inserted during the photobleaching (decreasing) phase of the illumination cycle, we observed no additional decrease in fluorescence during this pause (data not shown). This indicates that the decrease in fluorescence is not due to leakage of the dye from the cell, but depends on the continuous illumination of the sample. It is conceivable that a dye leakage could be light-sensitive and that this process occurs only during periods of illumination; however to the best of our knowledge, there exists no prior evidence for such a mechanism. Therefore, we propose that the fluorescence attenuation observed in this experiment is mainly due to photobleaching of the fluorophores by intensive illumination.

We next observed a similar effect using BCECF, a commonly used pH indicator. As observed with the Ca^{2+}-reponsive dyes, under continuous illumination the BCECF-loaded sperm rapidly ceased to be motile, presumably due to ROS generation. Interestingly, we again observed decreasing fluorescence (due to photobleaching of BCECF) once the sperm had stopped moving (Figure 5 and supplementary material). In other words, the fluorescence intensity was almost constant as

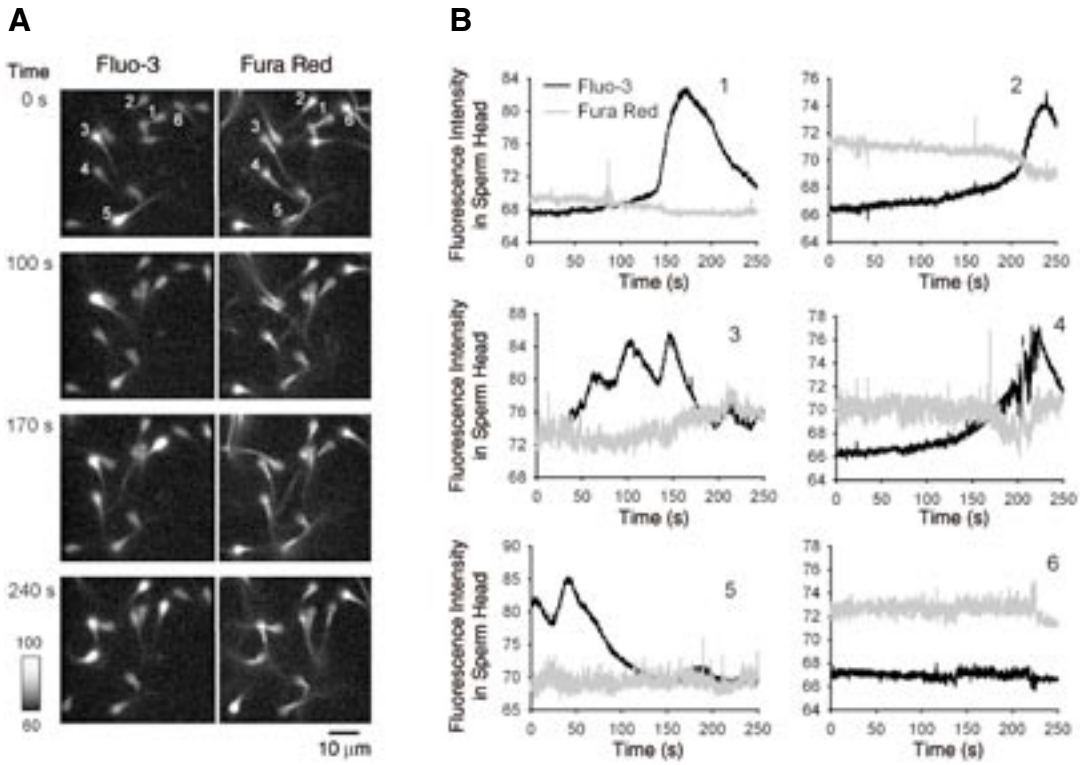

Figure 3. Ca^{2+} imaging with two different indicators using an image splitter. The decrease in fluorescence intensity of the Ca^{2+} indicator (fluo-3) observed in Figure 2 might be caused by photobleaching of the fluorophores or by a decrease in Ca^{2+} concentration ($[Ca^{2+}]i$). (A) Fluorescence images generated by pulsed illumination. Utilizing an image splitter as described in the Materials and Methods section, fluorescence signals from fluo-3 and Fura Red were captured simultaneously. (B) Kinetics of fluorescence intensity in sperm heads. Fluo-3 (black) and Fura Red (gray) fluorescence intensities from six individual sperm head regions were plotted. As seen in Figure 2, fluorescence fluctuations were observed using both indicators. The direction of the fluorescence changes of the two indicators were opposed, indicating that in this system they reflect a real change in the $[Ca^{2+}]i$. The results are representative of three experiments. The movie images are available in the supplementary material (Supplementary Movie 3).

long as sperm kept moving. In LED pulsed illumination system, the sperm kept moving with constant fluorescent signals for a longer period compared with the continuous illumination system. However, pulsed illumination by the LED for extended periods was also sufficient to inhibit sperm motility and cause photobleaching of the fluorophore (Figure 6), indicating that BCECF is more susceptible to ROS generation than fluo-3. As was observed under continuous illumination, photobleaching of BCECF by the LED commenced once the sperm had stopped moving (Figure 6).

DISCUSSION

Changes in $[Ca^{2+}]i$ are believed to play a fundamental role in controlling sperm flagellar wave form. A direct understanding of the relationship between sperm $[Ca^{2+}]i$ and its flagellar form requires measurement of Ca^{2+} changes in a beating flagella. However, capturing clear fluorescence images from a rapidly moving organelle such as sperm flagellum is technically challenging. In a pioneering study using a xenon stroboscope as fluorescence excitation light source, Suarez and coworkers (10) measured $[Ca^{2+}]_i$ changes in the beating flagella of hamster sperm. However the usefulness of this technique is limited due to large electromagnetic pulses generated by the xenon strobe lamp interfering with other electronic devices. In 2003, Wennemuth et al. (11) used an LED as a brightfield stroboscopic light source to study sperm flagellar wave form. Their success encouraged us to develop a LED-based stroboscopic light source for use in fluorescence microscopy. We utilized a high power LED to perform Ca^{2+} imaging of swimming sea urchin sperm for the first time and discovered the relationship between sperm flagellar form and its $[Ca^{2+}]i$ is more complex than had been previously proposed (8).

One of the potentially beneficial characteristics of an LED-based illumination system, apart from the ability to "freeze" the motion of rapidly moving objects such as the sperm flagellum, is that the illumination of the sample is reduced to short pulses of light (0.5–2 ms for our experiments). This reduces the production of ROS to a minimum, an important factor in preventing phototoxicity during the course of extended experiments. We have shown in this study that LED-based illumination greatly diminishes phototoxicity compared with continuous illumination with a mercury lamp. We also demonstrated reduced photobleaching of a number of fluorophores under the same conditions, which together with reduced phototoxicity enables experiments to be performed over longer time scales than with conventional epifluorescence illumination.

One way to reduce phototoxicity and photobleaching with mercury or xenon lamp illumination is to use a mechanical shutter to block excitation light between successive images. However, there is always a time delay to open or close a shutter completely (e.g., 3 ms to open the 25-mm diameter *UNIBLITZ* VS25 shutter), and it is difficult to reduce the pulsed illumination time to the submillisecond range possible with an LED. Furthermore, as

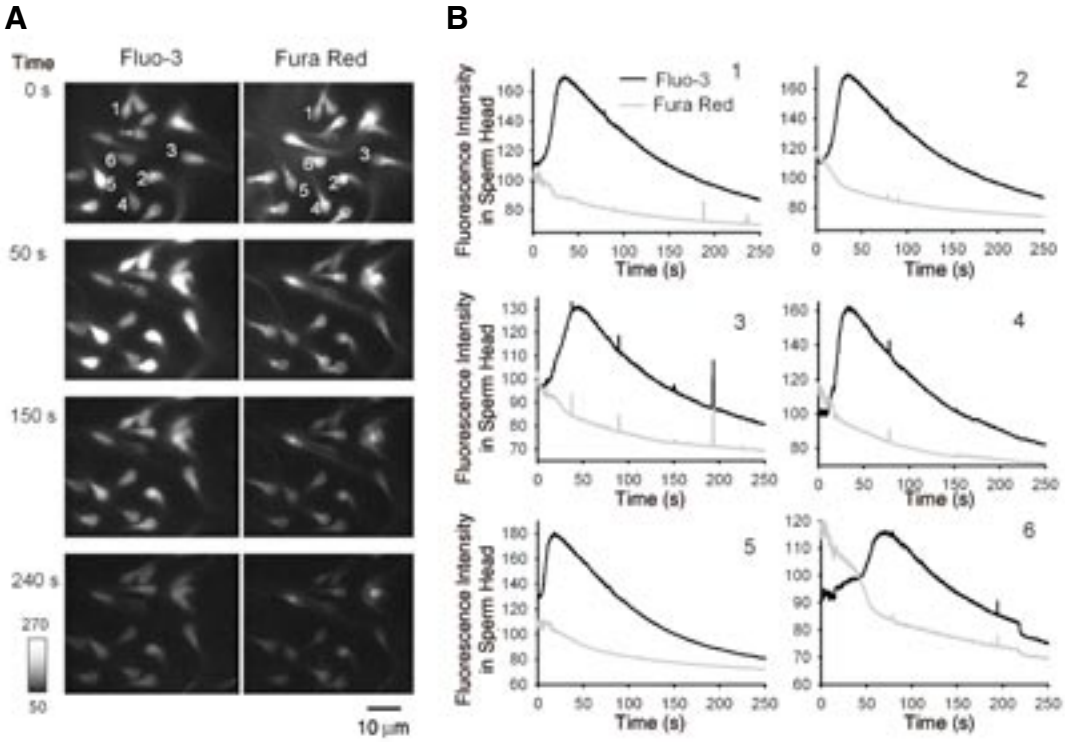

Figure 4. The decrease in the fluorescence intensities caused by the continuous illumination system is due to photobleaching of the Ca^{2+} indicator. (A) As in Figure 3A, but illuminated continuously with the mercury lamp. (B) As Figure 3B, but illuminated continuously with the mercury lamp. When the signal of fluo-3 decreased after reaching a maximum level, the Fura Red signal did not increase; instead, it slowly decreased, indicating that the fluorescence intensity changes in this phase do not wholly reflect a change in Ca^{2+} concentration ($[Ca^{2+}]i$). The results are representative of three experiments. The movie images are available in the supplementary material (Supplementary Movie 4).

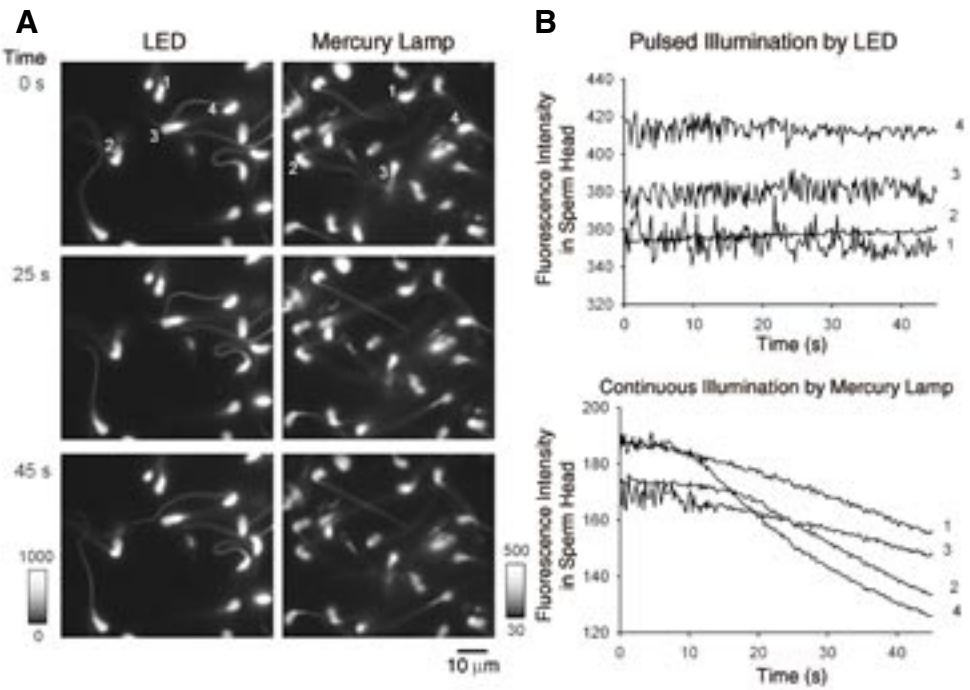

Figure 5. Human sperm pH imaging performed with light-emitting diode (LED)-based pulsed illumination and continuous illumination with a mercury lamp. As described in Figure 2, pH imaging was performed with human sperm loaded with BCECF using two different illumination systems. (A) Fluorescence images of human sperm were expressed using a gray-scale. Most of sperm illuminated by pulsed light (LED) remained motile during the entire experiment, while sperm illuminated continuously (mercury lamp) ceased moving around 10 s after the start of illumination. (B) Kinetics of fluorescence intensity changes in sperm heads in two different illumination systems. Fluorescence intensities of four individual sperm (head region) were plotted in each system. In the LED system, the fluorescence signals (intracellular pH) were basically constant. On the other hand, a decrease was observed in the fluorescence signals when using the continuous illumination system. Notably, when sperm stopped moving, the decrease in fluorescence intensity accelerated. The results are representative of more than 10 experiments. The movie images are available in the supplementary material (Supplementary Movie 5).

shutters open and close, they generate vibrations that can degrade the quality of images collected and generate heat that, when driven very hard for extended periods, can easily lead to failure of the equipment. Therefore, an LED-based pulsed illumination system offers significant advantages in reducing the total illumination time for examination of specimens on a fluorescent microscope.

It is worth mentioning that we applied 3 A DC pulse current to the LED to generate high intensity light, whose momentary energy can be estimated up to 27 W (27 mJ at 1-ms pulse). This value is about five times higher than that of the standard usage in a continuous illumination mode (700 mA, 5 W) according to the data file of the product (Lumileds Lighting LLC).

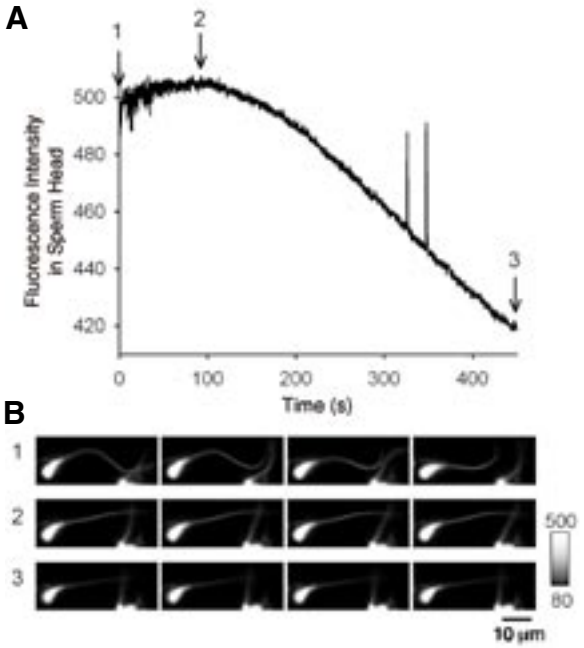

Figure 6. Pulsed illumination by light-emitting diode (LED) can also cause cytotoxicity and photobleaching of BCECF. Sperm pH imaging by pulsed illumination was performed as in Figure 5, but for an extended period. (A) Representative record of the kinetics of BCECF fluorescence intensity from a sperm head. The fluorescence intensity was constant up to 100 s, but declined afterwards. (B) Series of fluorescence images of the BCECF-loaded human sperm. Sequences of single sperm fluorescence images (four images at 0.2-s intervals) were selected at the times indicated by arrows in panel A. The second sequence of images indicates that the sperm ceased moving, but the fluorescence intensities were unchanged (upper images). The results are representative of more than 10 experiments. The movie images are available in the supplementary material (Supplementary Movie 6).

Therefore, a pulsed illumination mode of the LED can produce brighter light than a continuous illumination mode. We confirmed that the excess current in the pulse mode does not damage the LED as long as the integrated illumination time is <10% of the entire period (10% duty cycle). For example, 1-ms pulses at 100 Hz or 2-ms pulses at 50 Hz result in 100 ms total illumination during 1 s, which is equivalent to 2.7 W. To our knowledge, our system generates the brightest illumination among previously reported LED-based illumination systems for fluorescence imaging (12–14). It should be noted that the 100 W mercury lamp still generates brighter excitation light than the pulsed-LED system in our experiments. In this study, we had to reduce the intensity of the mercury lamp light using the beam splitter (80% reduction) to make both systems comparable.

Besides the advantages of pulsed illumination described above, LEDs have other advantages over mercury or xenon lamps, as mentioned in previous studies (12,13,15): (*i*) small size; (*ii*) "instantaneous" illumination; (*iii*) low energy requirements (<5 W); (*iv*) safety (no risk of explosion nor release of toxic material); (*v*) relatively inexpensive; (*vi*) long life (up to 100,000 h); and (*vii*) stable light emission (14). One shortcoming is a lack of high-power LEDs in the UV range, however, some reports of the development and application of higher power UV LED light sources have recently appeared (16,17). Considering all the above, we believe that LEDs will increasingly replace mercury and xenon lamps in future fluorescence cell imaging studies.

In this study, we found that a sustained increase in sperm $[Ca^{2+}]i$ together with reduction of sperm motility occurred as a consequence of phototoxicity. Sperm, as well as other live cells, use multiple types of Ca^{2+} transporters such as Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers to regulate the $[Ca^{2+}]i$ and maintain it low (18). If some of these transporters lose their function due to ROS, the $[Ca^{2+}]i$ might increase independently of the activation of Ca^{2+}-permeable channels. In addition, there is accumulating evidence indicating that several types of Ca^{2+}-permeable channels are regulated by the redox state (19). Therefore, the $[Ca^{2+}]i$ increase observed in this study is possibly caused by both reduction of the Ca^{2+} clearance capability and Ca^{2+} influx or Ca^{2+} release through Ca^{2+}-permeable channels.

We also report some interesting aspects of photobleaching of fluorescence indicators during cell imaging. We expected that photobleaching would increase gradually and continuously from the first exposure to excitation light. However, photobleaching was minimal the entire time that the sperm remained motile but rapidly accelerated when sperm motility ceased during both Ca^{2+} and pH imaging. It is known that the mitochondria of mammalian sperm generate endogenous ROS through electron leakage from complex 1 and 2 of the respiratory chain (20) and that sperm motility decreases markedly in the presence of exogenous ROS (21). It seems plausible that sperm posses ROS scavengers that protect mitochondria from damage, but that in the presence of excess ROS, in our case by the excitation of fluorophores, the scavengers become exhausted and mitochondrial damage ensues. Once the putative ROS scavengers are depleted and the concentration of ROS increases, photobleaching accelerates, possibly assisted by an increase in cellular oxygen levels due to the decrease in mitochondrial activity. Hence, the dual phenomena of loss of motility and photobleaching may be synergistically linked. One way to test this in the future would be to see if exogenous scavenger molecules such as catalase and/or dimethylsulphoxide (21) could prolong the duration of sperm motility while delaying the acceleration of photobleaching under continuous illumination.

In this study we clearly demonstrate the advantages of LED-based stroboscopic illumination systems for fluorescence cell imaging using human sperm. Combined with the other advantages also outlined, we expect it will become an increasingly popular technique for fluorescence imaging in the future.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Takuya Nishigaki, Instituto de Biotecnologia, UNAM, Cuernavaca, Morelos 62210, Mexico. e-mail: takuya@ibt.unam.mx