Int J Legal Med
DOI 10.1007/s00414-017-1536-3

ORIGINAL ARTICLE

Advanced forensic validation for human spermatozoa identification using SPERM HY-LITER™ Express with quantitative image analysis

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Received: 29 August 2016 / Accepted: 10 January 2017

Abstract Identification of human semen is indispensable for the investigation of sexual assaults. Fluorescence staining methods using commercial kits, such as the series of SPERM HY-LITER™ kits, have been useful to detect human sperm via strong fluorescence. These kits have been examined from various forensic aspects. However, because of a lack of evaluation methods, these studies did not provide objective, or quantitative, descriptions of the results nor clear criteria for the decisions reached. In addition, the variety of validations was considerably limited. In this study, we conducted more advanced validations of SPERM HY-LITER™ Express using our established image analysis method. Use of this method enabled objective and specific identification of fluorescent sperm's spots and quantitative comparisons of the sperm detection performance under complex experimental conditions. For body fluid mixtures, we examined interference with the fluorescence staining from other body fluid components. Effects of sample decomposition were simulated in high humidity and high temperature conditions. Semen with quite low sperm concentrations, such as azoospermia and oligospermia samples, represented the most challenging cases in application of the kit. Finally, the tolerance of the kit against various acidic and basic environments was analyzed. The validations herein provide useful information for the practical applications of the SPERM HY-LITER™ Express kit, which were previously unobtainable. Moreover, the versatility of our image analysis method toward various complex cases was demonstrated.

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Keywords Semen identification · Sperm · Fluorescence staining · SPERM HY-LITER · Fluorescence image

Introduction

In crime scenes involving bodily violence, body fluids are frequently left and then collected as evidence. Identification of body fluids offers critical information to investigate how the crimes actually occurred. In investigations of sexual assaults, identification of human semen on skin or clothes, or in the vagina of the victim, is indispensable.

Identification of human semen has been conducted by several enzymatic or serological methods [1, 2]. These methods detect characteristic components in semen, such as prostatic acid phosphatase, prostate-specific antigen and semenogelin by colorimetric assays, immunochromatography, and enzyme-linked immunosorbent assays (ELISAs) [1–10]. A combination of chemical staining and subsequent microscopic observation is also used to detect spermatozoa [11–14]. The characteristic colors enable the distinction of sperm based on their round heads and tails. The current methods provide pragmatic convenience to suggest the presence of semen. However, there have been reports that these methods have cross-reactivity to other body fluids [1, 2, 15, 16]. In addition, microscopic observation with chemical staining can be strongly hindered by coexisting materials, degradation of the sperm's morphological structure, and detachment of sperm's tail [15–17].

A novel alternative method is a fluorescence staining method that targets antigen proteins on the head of human sperm. Currently, useful fluorescence staining kits are available, including a series of SPERM HY-LITER™ kits (Independent Forensics, Lombard, IL, USA). These kits use an antibody that stains the head of human sperm specifically with a green

Published online: 19 January 2017

fluorescent dye; another fluorescent dye is used to visualize nucleic acids [18–20]. These kits have a remarkable advantage to identify spermatozoa because of their easy detection with strong fluorescence and robustness against low sperm concentration and surrounding materials.

There have been several reports of validation analyses for SPERM HY-LITER™ kits from various forensic aspects, such as specificity, sensitivity, and robustness against contamination [15–18]. However, because they detected fluorescent sperm's spots by visual observation, the experimental results could not be discussed objectively nor quantitatively. Then, the variety of the validation has been quite limited.

Consequently, in our previous study, we developed an image analysis method to automatically detect light spots and quantify them [21]. This method uses Laplacian differentiation of fluorescence images, which enables the sensitive detection of the spot's edge. In addition, structural criteria were established for the specific identification of sperm's light spots, based on their area and circularity. This image analysis method and criteria offer an innovative advantage for the objective and specific identification of sperm spots over the previous way by visual observation. In addition, the use of this method and criteria enables quantitative evaluation of the efficiency of sperm detection or sperm staining in any experiment, which could not be achieved previously.

In this study, we performed more advanced validations of the SPERM HY-LITER™ Express kit, quantifying the sperm detection performance in each experimental condition using our image analysis method and criteria. The analysis of actual crime samples can be more confusing compared with those assessed in previous studies; therefore, further studies of more complex conditions or the provision of more accurate information are urgently needed. The validations herein include those for degraded samples, low sperm samples, mixtures of semen and other body fluids, and exposure to acid and basic solutions. Such difficult samples are often found in actual crime scenes, representing challenges to forensic examiners. Detailed and systemized interpretations of the applicability of the kit to such complex cases would benefit its practical use. In addition, this study demonstrated the versatility of the quantification method for various experimental aims.

Materials and methods

Collection of body fluids and preparation of body fluid traces

Body fluid samples (semen, saliva, urine, blood, and vaginal fluid) were collected from six (for semen) or five (for saliva, urine, blood, and vaginal fluid) Japanese volunteers aged 20 ~ 30. Vaginal fluid was obtained using a sterile cotton swab (ø 12 mm) and wiped from the vaginal wall. The collected samples were all stored at −80 °C until assayed. Semen samples of azoospermia individuals were selected by microscopic observation, which resulted in absence of sperm. To make a semen trace, 100 μL of semen was spread on a piece of white cotton cloth and dried at room temperature overnight. For the degradation assay, the semen trace on the cotton cloth was cut into 5 × 5 mm pieces. The semen trace pieces were separately put into plastic containers in which a paper towel wetted with distilled water was spread on the bottom. The plastic containers were stored at 37 °C for 2 weeks, 1, 2, and 3 months, respectively. For the mixture staining assay, 3 μL of semen and 3 μL of saliva, urine, or blood were mixed in a microtube. Each mixture was then spread on a 5 × 5 mm piece of cotton cloth. Traces of semen and vaginal fluid were prepared by adding 3 μL of semen on a 1/32 piece of a cotton swab with vaginal fluid. These traces with body fluid mixtures were dried and stored under ambient conditions for 1 or 2 days. In the validation study for acid and base solutions, 50 mM phosphate solutions at pH 1, 3, 5, 7, 9, 11, or 13 were prepared. Three microliters of semen and 10 μL of each phosphate solution were added onto a 5 × 5 mm piece of cotton cloth. The semen traces were dried overnight.

Fluorescence staining

Each 5 × 5 mm piece of cotton cloth with body fluid, or each piece of a cotton swab with semen and vaginal fluid, was extracted with 150 μL of distilled water by pipetting. The body fluid extracts were fixed on a glass slide and stained using SPERM HY-LITER™ Express, according to a protocol described in our previous report [21]. Baecchi staining in "Degradation in high humidity and high temperature" section was also conducted to the fixed semen extract as explained in our previous report [21].

Microscopic observation

Fluorescence images of each stained sample were acquired using an upright microscope (Axio Imager M1, Carl Zeiss, Oberkochen, Germany), equipped with a 20× Zeiss Apochromat dry objective (NA 0.8). The filter sets for imaging 4′,6-diamidino-2-phenylindole (DAPI; nuclei) and CF™ 488A (green fluorescence; sperm heads) were λ_{Ex} (365 nm)/ λ_{Em} (445/50) and λ_{Ex} (470/40)/ λ_{Em} (520/50), respectively. Differential interference contrast (DIC) images were obtained consecutively. The microscopic system was operated by Axio Vision SE64 software (Carl Zeiss).

Image analysis and statistics

For the quantitative evaluation of fluorescently stained sperm, the CF™ 488A fluorescence images were analyzed by a Laplacian and Gaussian (LOG) method that was developed

in our laboratory [21]. This image analysis method uses the second derivative images for automatic detection of a sperm's light spot and characterizes the spot by area, mean fluorescence intensity, and circularity (Fig. 1 in reference no. 21). Based on previously established criteria, light spots with ≥10.3 μm^2 in area and ≥0.307 in circularity were identified as positive for human sperm. The efficiency of sperm detection was defined as the percentage of positive spots among all the spots detected (≥2.0 μm^2 in area) by the LOG method.

In "Degradation in high humidity and high temperature" section, temporal changes of both sperm detection efficiencies and relative fluorescence intensities were examined statistically by Scheffe's F test, preceded by Bartlett and Kruskal–Wallis tests. Fluorescence intensities in "Body fluid mixtures" section were compared using the F test and t test. In "Tolerance against acids and bases" section, the effects of acid and basic solutions on sperm detection efficiency were analyzed by Scheffe's F test, following Bartlett and Kruskal–Wallis tests. Differences in fluorescence intensities were examined by the Bartlett test and single-factor ANOVA.

Results and discussion

Degradation in high humidity and high temperature

To examine effects of degradation of semen traces on sperm detection using SPERM HY-LITER™ Express, we incubated the six semen traces in highly humid conditions at 37 °C for 2 weeks to 3 months. The degraded traces and fresh ones (stored for 1 day in ambient condition) were stained using the kit, and the fluorescence images were acquired by fluorescence microscopy observation (Fig. 1a). Subsequently, the mean fluorescence intensity of the light spots detected and the sperm detection efficiencies were evaluated by the LOG method. The mean fluorescence intensities decreased gradually with time, as did the sperm detection efficiencies (Fig. 1b, c). At 2 weeks of incubation, the sperm detection efficiency decreased significantly (56.7%) compared with that in the fresh samples (80.4%); nonetheless, a decrease in the fluorescence intensity was not detected. This result suggested that the morphological structures of the sperm's spots (area and circularity) were partially distorted. Indeed, some small light spots were visualized in the CF™ 488A images, which could be attributed to fragmented sperm. This may be because the sperm heads had collapsed or became more fragile during incubation. After incubation for 1 month, the decomposition of sperm heads proceeded further, and the detection efficiency fell to 28.3%. The fluorescence intensity also significantly decreased, which was considered to reflect the degradation of the protein antigens on the sperm head. After incubation for 2 months, identification of sperm was almost impossible (1.2%) and reached zero after 3 months. Figure 1d represents bright-field images of the degraded semen traces stained by Baecchi staining method, one of the popular chemical staining methods for forensic sperm identification. After incubation for more than 2 weeks, it was almost impossible to find sperms to which their tails were attached. Moreover, after more than 2 months, it was very hard to find objects which looked like sperm's head, corresponding to the results by the SPERM HY-LITER™ Express.

The LOG method detected fragmentation of the sperm head sensitively, reflecting the decrease of sperm detection efficiency, that is, the increase of detection of small and distorted spots. The fragmentation of the sperm head was also observed in aged semen traces that were stored in ambient condition for 30 years [21]. However, the light spots on the fragmented sperm are not useful for human sperm identification because such small spots can be found in other body fluid samples, which are mainly derived from autofluorescence. The present results demonstrated that degradation causes not only protein degradation but also distortion of the sperm's shape. Thus, the use of the structural criteria for light spots is indispensable for reliable sperm identification in degraded samples.

Semen samples with low sperm concentration

Semen with a low sperm count, attributed to oligospermia and azoospermia, represents one of the most confusing cases of semen identification in crime investigations. In particular, the symptoms of azoospermia appear in 1% of the male population and cause absence of sperm in semen [22, 23]. We prepared four traces of azoospermia semen samples in which no sperm was found by preliminary microscopic observation with chemical staining. Subsequently, the semen traces were stained using SPERM HY-LITER™ Express. Figure 2 shows the fluorescence images obtained. Most view fields on the sample plate were completely dark and showed no sperm. However, in two of the four azoospermia samples, one or two bright light spots were observed on the CF™ 488A image. Image analysis using the LOG method and the structural criteria recognized these spots as sperm-positive. This is the first report to demonstrate sperm identification in azoospermia samples using a fluorescence staining kit, indicating its superiority over chemical staining techniques. In this experiment, the LOG method was practically efficient to detect these few sperm, because it offered automatic and fast screening of fluorescence images from a large view field. Moreover, the structural criteria for sperm spots strongly supported the view that the spots actually represented sperm, even without their tails. The combination of the fluorescence staining kit and the image analysis method enabled efficient and reliable sperm identification in semen samples with a very low sperm concentration.

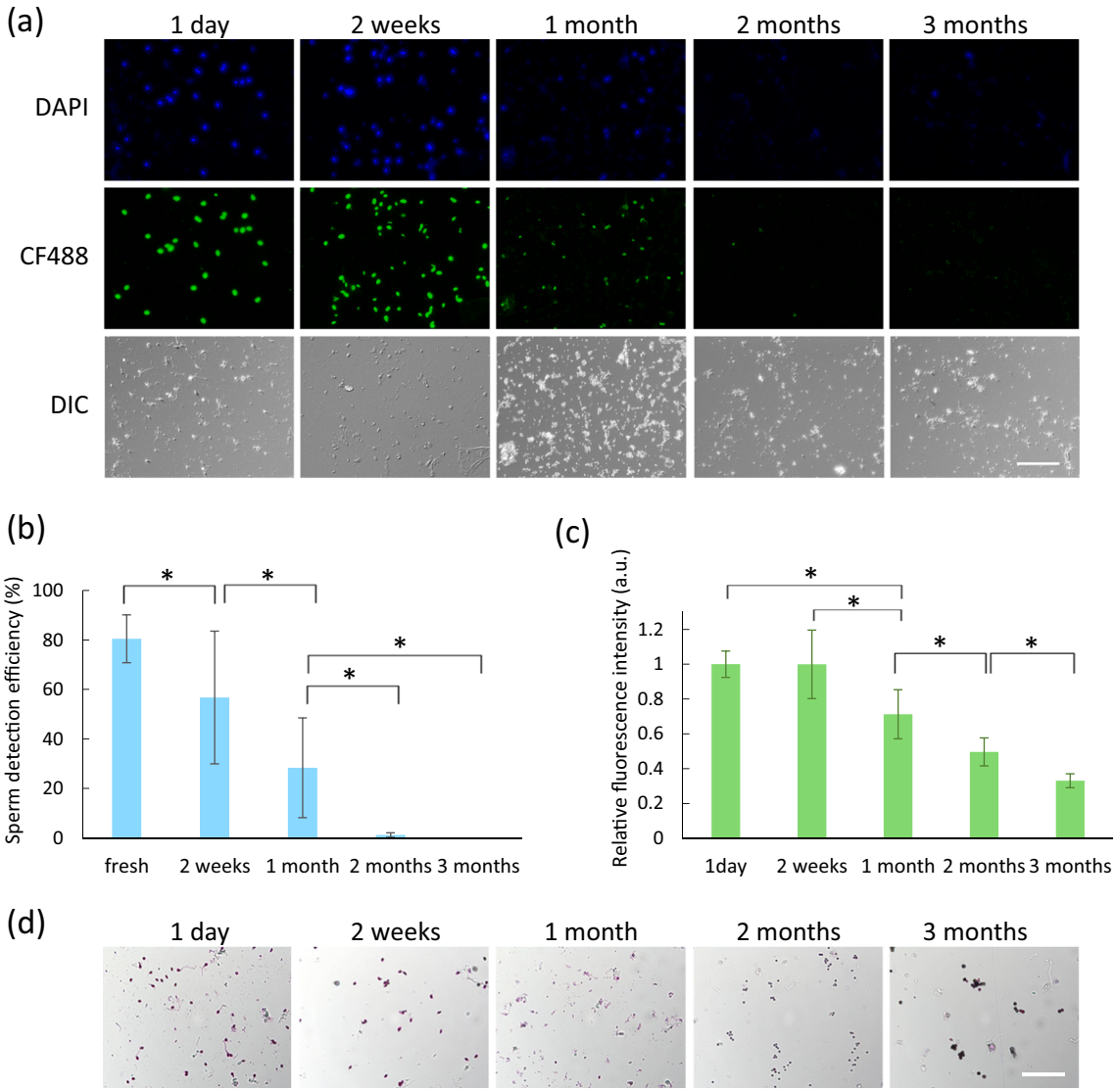

Fig. 1 Fluorescence staining of degraded semen traces. **a** Fluorescence images of fresh and degraded semen traces, which were incubated in humid conditions for different periods. The *scale bar* represents 50 μm. **b**, **c** Mean fluorescence intensities (**b**) and sperm detection efficiencies (**c**) in fluorescence staining of fresh and degraded semen traces (*$p < 0.05$). *Error bars* indicate 90% confidence intervals ($n = 6$). **d** Bright-field images of fresh and degraded semen traces stained by the Baecchi staining method. The *scale bar* represents 50 μm

Body fluid mixtures

In actual samples from sexual assaults, semen is often found as a mixture with other body fluids, usually vaginal fluids. Thus, sperm needs to be detected even if materials from other body fluids contaminate the samples. We collected five samples each of blood, saliva, urine, and vaginal fluid and four semen samples. We then prepared four mixture traces of semen with each of the other body fluid sample (80 mixture traces in total) and four traces of pure semen ($n = 4$ in each mixture or pure trace case). Each trace was stained fluorescently with SPERM HY-LITER™ Express, and the sperm were observed under a fluorescence microscope. As shown in the DIC images, sperm were surrounded by many materials from other body fluids (Fig. 3a). In particular, epithelial cells in saliva and vaginal fluid covered the sperm. Such conditions made it quite difficult to find sperm. However, in the CF™ 488A images, the surrounding materials exhibited no significant fluorescence and did not shield fluorescence from the sperm. Thus, the stained sperm could be clearly observed. The LOG image analysis system not only detected fluorescence from sperm spots but also from many small light spots. Such small spots were derived mainly from weak autofluorescence of coexisting body fluid components [21]. Thus, the total number of light spots was influenced strongly by the amount of surrounding materials. To evaluate the visualization of sperm, we compared the mean fluorescence intensities of sperm spots (positives) (Fig. 3b). The statistical analysis

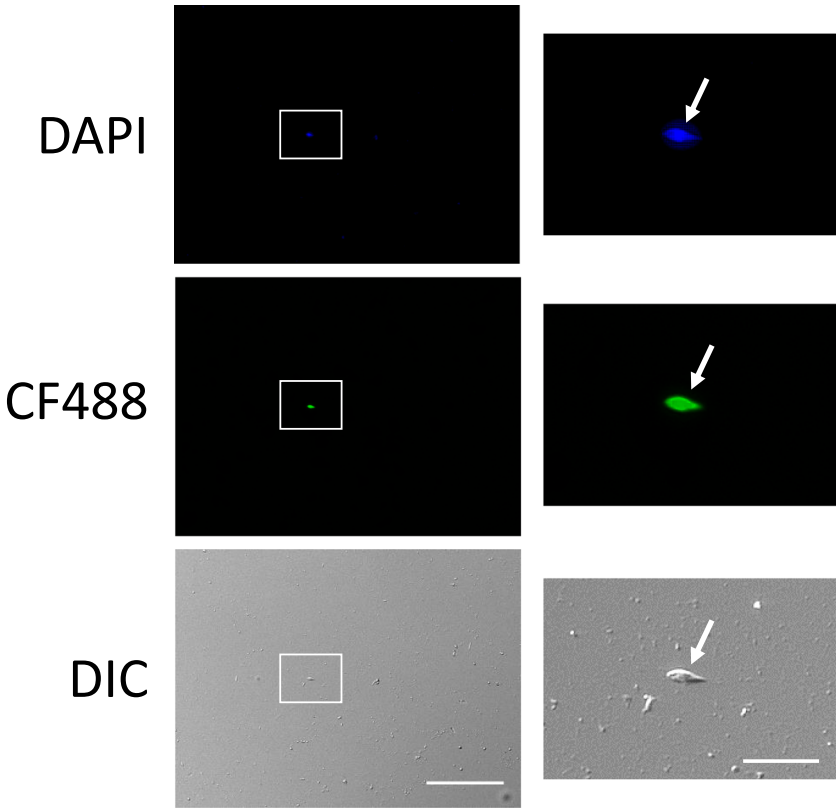

Fig. 2 Sperm identification in azoospermia. Fluorescence images obtained from azoospermia semen samples. Those on the right are magnified images of the squared regions on the left. *Arrows* point to a sperm's light spot. The *scale bars* represent 100 μm (*left*) and 20 μm (*right*)

showed that most of the mixed samples showed no significant change in fluorescence intensity compared with the pure semen sample. Meanwhile, two blood mixtures showed higher fluorescence intensities than the pure semen samples ($p < 0.05$). We considered that this might be caused by strong autofluorescence in blood samples, which increased the whole fluorescence level in the images. Consequently, the use of SPERM HY-LITER™ Express and our image analysis methods detected the sperm successfully, even in mixtures with other body fluids. In addition, the quantification of the staining efficiency clearly verified that there was no negative interference from the other body fluids on the visualization of sperm.

Tolerance against acids and bases

Acidity or alkalinity is one of the factors that inhibit chemical reactions. Processes using fluorescent staining

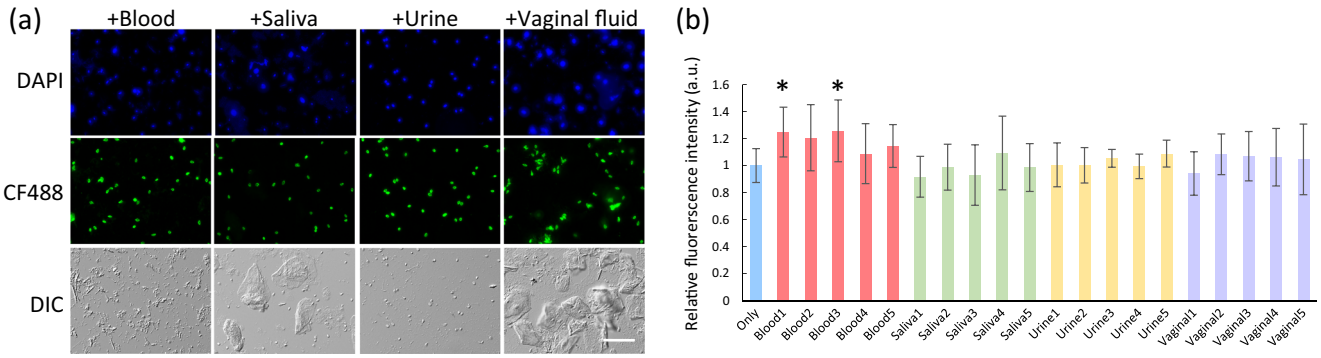

Fig. 3 Validation of semen mixtures with other body fluids. **a** Fluorescence images of mixed traces of semen and various other body fluids. The *scale bar* represents 50 μm. **b** Relative fluorescence intensities of sperm light spots identified in the mixed samples, setting the mean intensity in the pure semen sample as 1 (*$p < 0.05$, significantly different compared with that of semen only). *Error bars* indicate 90% confidence intervals ($n = 4$)

involving protein interactions generally prefer moderate conditions to maintain protein reactivity. Thus, the use of buffers is indispensable. Unfortunately, forensic samples can be exposed frequently to invasive situations with quite low or high pHs, because many materials, products, and foods encountered in daily life have acid or basic properties. In this experiment, we examined the robustness of the fluorescence staining with SPERM HY-LITER™ Express in acidic or basic environments. We prepared seven solutions with pH from 1 to 13 and spread each of them on four semen traces (n = 4 in each pH condition). The semen traces were directly extracted and stained using the kit in the usual way. Figure 4a represents the fluorescence images of the stained traces. Despite the sperm being soaked in strong acid or base, they retained their heads with the round structure and their tails attached, as shown in the DIC images. In addition, sperm were visualized successfully in both the CF™ 488 and the DAPI images. The CF™ 488 images were then analyzed by the LOG method, including evaluation of the fluorescence intensities of the light spots and the sperm detection efficiencies. The mean fluorescence intensities did not show significant changes among the seven experimental conditions (data not shown). This result indicated that protein antigens on the sperm were not denatured significantly by strong acids or bases. In addition, no negative interference in the antibody-antigen interaction was identified. The sperm detection efficiency also demonstrated strong tolerance against acidity (Fig. 4b). No change was observed after exposure to strong acid compared with neutral pH conditions. Meanwhile, a significant decrease in the sperm detection efficiency was detected after exposure to the pH 13 solution compared with the other conditions. In the CF™ 488 image of the pH 13-treated sample, some small, dispersed light spots were observed. The small spots may have resulted from fragmentation of sperm or partial detachment of sperm head constituents. An increase of the amount of such small spots led to a decrease in the percentage of positive sperm spots among total spots. Thus, use of the quantification method demonstrated the fluorescence staining kit's performance for semen samples exposed to various pH conditions. The LOG method detected the small spots sensitively and suggested the possibility of partial disruption of sperm constituents under strong alkaline conditions. Such alkaline conditions include, for example, exposure to cleaners containing lyes, concrete, and luminol reagent in forensic investigation [24].

Conclusion

In this study, we used the SPERM HY-LITER™ Express kit for advanced forensic validation tests. Quantification using our LOG method and structural criteria enabled detailed and clear comparisons of the staining performance under various conditions. For the degraded semen traces, morphological disruption of the sperm head was observed within 2 weeks. With further incubation and degradation, the sperm detection efficiency ultimately fell to zero at 3 months. The combination of SPERM HY-LITER™ Express and our image analysis method was highly effective to analyze semen traces with extremely low sperm concentrations, such as azoospermia semen samples. We verified that SPERM HY-LITER™ Express could detect sperm successfully in mixed body fluid samples. Visualization of sperm was not hindered by components from other body fluids. Finally, SPERM HY-LITER™ Express showed a robust performance for semen samples exposed in acid conditions. Meanwhile, our results suggested that the sperm's structure was disrupted under strong alkaline conditions.

The use of our LOG method and structural criteria expanded the range of the available validations and demonstrated the applicability of the kit to several challenging cases. The information provided here will promote efficient and reliable sperm identification using both the kit and the image analysis method in actual forensic analysis.

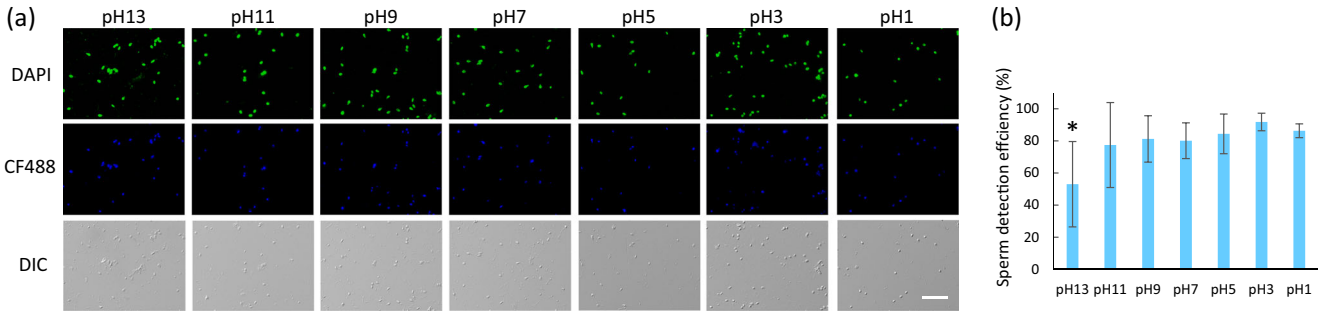

Fig. 4 Assessment of tolerance to acids and bases. **a** Fluorescence images of semen traces exposed to various acids and base solutions. The *scale bar* represents 50 μm. **b** Sperm detection efficiencies of semen traces under various pH conditions (*p < 0.05). *Error bars* indicate 90% confidence intervals (n = 4)

Compliance with ethical standards All procedures involving human participants were approved by the Institutional Review Board of the National Research Institute of Police Science (Kashiwa, Japan).

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