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ORIGINAL ARTICLE

Sperm Biology

Antigen unmasking does not improve the visualization of phospholipase C zeta in human spermatozoa

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Phospholipase C zeta (PLCζ) is a sperm-specific protein that triggers oocyte activation. The analysis of PLCζ expression in human spermatozoa can be used as a diagnostic marker for oocyte activation deficiency. Our laboratory has previously optimized a standard "in-house" assay to determine PLCζ expression in human spermatozoa. However, one study has suggested that an antigen unmasking method (AUM) would be more efficient in visualizing PLCζ in human sperm. This study aimed to compare our established assay and AUM (involving HCl, acidic Tyrode's solution [AT], and heat). The mean relative fluorescence (RF) intensity of PLCζ in frozen-thawed spermatozoa from fourteen fertile donors stained with the in-house method was significantly higher than three other AUM groups (in-house [mean ± standard error of mean]: 18.87 ± 2.39 arbitrary units [a.u.] *vs* non-AUM: 11.44 ± 1.61 a.u., AT-AUM: 12.38 ± 1.89 a.u., and HCl-AUM: 12.51 ± 2.16 a.u., $P < 0.05$, one-way analysis of variance). The mean RF intensity of PLCζ in AT- and HCl-treated spermatozoa from 12 infertile males was not significantly different from that of the non-AUM group. However, the in-house method resulted in the highest RF intensity (12.11 ± 1.36 a.u., $P < 0.01$). Furthermore, specificity testing of antibody-antigen binding indicated that the in-house method showed more specific binding than spermatozoa treated by the AUM. In conclusion, our in-house method showed superior visualization and reliability than the AUM, thus supporting the continued use of our in-house assay for clinical research screening.

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INTRODUCTION

Upon sperm fusion, the fertilized oocyte undergoes a series of events that are collectively referred to as "oocyte activation", including maternal messenger ribonucleic acid (mRNA) degradation, pronuclei development, and gene and protein synthesis.[1] This spatiotemporal process marks the formation of an embryo.[2] The mechanism underlying mammalian oocyte activation is a series of calcium ion (Ca^{2+}) oscillations due to Ca^{2+} release from the endoplasmic reticulum after activation.[3]

Assisted reproductive treatment (ART), including conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), has improved pregnancy outcomes for many infertile or subfertile couples. However, fertilization failure remains a common problem in ART, affecting 5%–10% of IVF and 2%–4% of ICSI cases.[4,5] In addition, total fertilization failure, in which oocytes fail to become fertilized, occurs in 1%–3% of ICSI cases; this is often due to oocyte activation deficiency (OAD).[6]

Substantial evidence has shown that phospholipase C zeta (PLCζ) plays a crucial role in initiating Ca^{2+} oscillations and oocyte activation.[7,8] PLCζ is a sperm-specific protein that is expressed in the sperm head.[8] A subsequent research study showed that microinjections of sperm extract containing PLCζ into mouse oocytes elicited Ca^{2+} oscillations that were similar to those that occur at fertilization.[9] Furthermore, the microinjection of PLCζ coding ribonucleic acid (cRNA), mRNA, and recombinant PLCζ protein, into oocytes has been shown to trigger Ca^{2+} release and oocyte activation.[10–12]

The crucial role of PLCζ in oocyte activation has been further illustrated by research revealing inadequate levels of Ca^{2+} release in mouse oocytes injected with human spermatozoa from subjects with previous ICSI failure,[12] while other studies have demonstrated a link between deficient PLCζ expression in human spermatozoa and OAD.[13,14] Moreover, infertile males exhibiting globozoospermia or teratozoospermia have been shown to express reduced levels of sperm PLCζ.[13,15]

Immunofluorescence analysis has further revealed that PLCζ is predominantly expressed in the equatorial segment of human spermatozoa but is also present in reduced amounts in the acrosome, postacrosomal region, or a combination of these locations.[16,17] Most notably, the relative fluorescence (RF) intensity of sperm PLCζ has been significantly and positively correlated with fertilization rates after ICSI,[18,19] although there is still debate relating to the exact mechanisms linking PLCζ expression and fertilization outcomes.[20] However, the current evidence suggests that PLCζ plays an important role in the biochemical pathways involved in male-factor infertility.

Our laboratory has optimized a standard "in-house" immunofluorescence staining protocol to visualize and evaluate

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PLCζ expression in human spermatozoa. This assay has been used consistently in a range of publications[14,16–18,21,22] and has been validated by several peptide-blocking experiments.[16,23,24] However, in one study, Kashir *et al*.[25] proposed that inadequate specificity between the antibody and the masked PLCζ antigens hindered visualization efficacy in PLCζ immunoanalysis. These authors incubated mouse, pig, and human spermatozoa with hydrochloric acid (HCl) or acidic Tyrode's solution (AT), or heated them with sodium citrate, and reported that these methods helped to unmask the antigens of PLCζ, thereby improving the RF intensity in PLCζ immunoanalyses. These authors further proposed that this antigen unmasking method (AUM) may help reduce the chance of misdiagnosed PLCζ deficiency in clinical settings. However, this study also showed that individual fertile donors exhibited variation in sperm PLCζ levels after AT-AUM.

Given the evidence attesting to the link between PLCζ deficiency and OAD, it is critical to identify the best method for assessing PLCζ levels in males suffering with infertility. The Human Fertilization and Embryology Authority (HFEA) recommends the use of artificial oocyte activation (AOA) only for patients with demonstrable evidence for OAD, such as PLCζ deficiency.[26] This guideline highlights the critical diagnostic need to identify the most accurate assay for evaluating PLCζ expression in the clinical setting. This study aimed to compare our in-house assay and the new AUM with regard to visualizing and quantifying PLCζ expression in spermatozoa from both fertile and infertile males.

PATIENTS AND METHODS

Patients

Frozen sperm samples from 14 donors were obtained from VivaNeo Sperm Bank (Düsseldorf, Germany). Fresh sperm samples were also obtained from 12 infertile from Oxford Fertility (Oxford, UK). All patients and donors provided written informed consent. The study was approved by the National Research Ethics Service (Research Ethics Committee, reference number: 10/H0606/65).

Participating infertile males from Oxford Fertility had to meet either one of the following eligibility criteria: (1) a low fertilization rate (<50%), total fertilization failure, or recurrent fertilization failure in previous ICSI cycles; or (2) abnormal sperm head morphology, including, but not limited to, grossly abnormal morphology, enlarged/pin-shape/diminished head, or 100% globozoospermic. Cases of severe oligozoospermia were excluded when the number of spermatozoa identified on the staining glass slide was fewer than 100 and thus insufficient for testing with PLCζ assay. Infertile males had previously been screened with in-house assay and showed significantly lower levels of "mean PLCζ levels" in their spermatozoa, and/or a significantly lower proportion of sperm exhibiting PLCζ than fertile donors. These two parameters can help identify infertile males who may benefit from AOA treatment.[27] Fertile controls included men with proven fertility who had (1) previously fathered a child via natural conception or (2) normal semen quality (5th WHO guidelines)[28] and had fertilized an oocyte in IVF cycles.

Sperm preparation

Fresh semen samples from fertile and infertile males were produced after at least three days of abstinence. Samples from 12 infertile males were acquired from Oxford Fertility and subjected to density gradient washing (DGW) and computer-assisted sperm analyses (CASA). The samples were incubated at 37°C to liquefy prior to processing.

Frozen sperm samples from the VivaNeo Sperm Bank were thawed before undergoing DGW. Sperm thawing was performed in accordance with the manufacturer's protocol. Subsequently, DGW involved 40/80 PureSperm medium (Nidacon International, Mölndal, Sweden) according to the manufacturer's protocol.[17] CASA was performed and then sperm were centrifuged (Eppendorf, Saxony, Germany) at 800*g* for 3 min followed by fixation with 4% (*w*/*v*) formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Excess fixative was then removed and fixed cells were stored at 4°C.

Antibody purification

A rabbit antihuman polyclonal antibody was produced with peptides derived from two human PLCζ amino acid sequences (MEMRWFLSKIQDDFRGGKI and CMNKGYRRIPLFSR; Villeurbanne, Lyon, France) and was synthesized by Cova-lab (Villeurbanne). The antibody was purified with the use of immunogenic peptides which can bind to an agarose-containing column, according to the manufacturer's instructions (SulfoLink Kit, Pierce Biotechnology, Rockford, IL, USA).

In-house immunofluorescence staining

Immunofluorescence staining was applied to fixed sperm samples from 3–4 individuals at the same time. Sperm concentration was assessed before immunofluorescence staining. In-house staining was then performed as previously described.[21] First, 50 μl of fixed sperm suspension was loaded onto 0.01% (*w*/*v*) poly-L-lysine-coated hydrophobic glass slides (Sigma-Aldrich). After 30 min, the cells were permeabilized with 0.5% (*v*/*v*) Triton X-100 in phosphate-buffered saline (PBS) overnight at 4°C. After three washes with PBS, the samples were incubated with 3% (*w*/*v*) bovine serum albumin (BSA; Sigma-Aldrich) in PBS at room temperature (RT) for 1 h, in order to prevent nonspecific antigen binding. Subsequently, spermatozoa were probed with 25 μg ml^{-1} fluorescein isothiocyanate (FITC)-tagged rabbit anti-PLCζ antibody in 0.05% (*w*/*v*) BSA-PBS and incubated overnight at 4°C. The glass slides were then washed three times with PBS and incubated with 5 μg ml^{-1} AlexaFluor-488-conjugated goat antirabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at RT. The glass slides were then washed three times with PBS and mounted with a medium containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

The glass slides were then observed at 40× magnification in a fluorescence microscope equipped with FITC filter (Eclipse 80i, Nikon, Tokyo, Japan), and images were captured with a camera (Nikon). Images were captured with a confocal microscope. Sperm samples were identified with a bright-field channel. Cell nuclei were identified by DAPI staining, and RF intensity of PLCζ was measured after 400 ms of exposure in the FITC channel. Multiple fields of view were captured, and the illumination settings remained the same for each experiment.

The AUM

The main difference between the AUM and our in-house method is that after permeabilization, one of three protocols was applied to unmask antigens, namely, HCl (1 mol l^{-1}), AT (pH 2.5–3) and heating (95°C) for 7 min with sodium citrate (10 mmol l^{-1}). After antigen unmasking (AU) treatment, 100 mmol l^{-1} Tris (pH 8.5) was applied to neutralize the spermatozoa. The reagents (HCl and AT) were the same as those in the AUM study.[25] Other differences between our in-house assay and the AUM are the BSA concentration used for blocking nonspecific antigens (3% and 5%, respectively, for 1 h) and the BSA concentration in PBS buffer used for antibody incubation (0.05% and 5%, respectively). Differences between the in-house and AUM staining methods are summarized in **Table 1**.

Table 1: Differences in the immunofluorescence staining of phospholipase C zeta in human spermatozoa with in-house and antigen unmasking protocols

Procedure	*In-house method*	*AUM*
AU	No	Cells received one of the following treatments HCl (1 mol l^{-1}, pH: 0.1–0.5) AT (pH: 2.5–3) Heating at 95°C for 7 min with sodium citrate at (10 mmol l^{-1}, 0.05% Tween 20, pH 6.0) Then, spermatozoa were neutralized with buffer (100 mmol l^{-1} Tris, pH 8.5)
Blocking agent	3% BSA	5% BSA
Blocking time (h)	1	1
Primary antibody	25 μg ml^{-1}, in 0.05% BSA-PBS	25 μg ml^{-1}, in 5% BSA-PBS
Second antibody	5 μg ml^{-1}, in 0.05% BSA-PBS	5 μg ml^{-1}, in 5% BSA-PBS
Fixation	4% formaldehyde	Methanol

The main differences between the in-house and AUM protocols are the uses of AUM reagents, and the BSA concentration for blocking unspecific antigens and antibody preparation. BSA: bovine serum album; HCl: hydrogen chloride; PBS: phosphate-buffered saline; AUM: antigen unmasking method; AU: antigen unmasking; AT: acidic Tyrode's solution

Specificity test

Peptide blocking was used to investigate antigen specificity. Both peptides with amino acid sequences corresponding to PLCζ sequences (C-RESKSYFNPSNIKE-coNH2 and C-ETHERKGSDKRGDN) were produced (Covalab, Lyon, France), which ensured that the anti-human PLCζ antibody would specifically bind to them. An excess of the two peptides (4 mg in total) was then incubated with BSA for 1.5 h on ice with vigorous shaking. All staining procedures in the peptide blocking assays were the same as those used in the immunofluorescence staining assays, except that the primary antihuman PLCζ antibody was diluted in BSA medium containing the peptides.

Study design

Four experiments compared the visualization of the in-house and AUM methods (**Table 2**). The comparative experiments used frozen spermatozoa from fertile donors (experiments 1 and 4) and fresh-fixed spermatozoa from infertile males (experiments 2 and 3). In experiments 1, 2, and 4, spermatozoa from an individual male were equally distributed for staining with the in-house, AT-AUM, HCl-AUM, and non-AUM methods. The non-AUM method refers to spermatozoa labeled following the AUM protocol in the absence of AUM reagents. According to Kashir *et al*.,[25] the most effective AUM involved heating with sodium citrate for 7 min, albeit no specific heating temperature was given in this publication. We chose heating at 95°C for 7 min but found that this damaged the spermatozoa and no fluorescence was observed (data not shown). In experiment 3, AUM reagents (HCl and AT) were used in the in-house method. Spermatozoa from every infertile/fertile individual were equally distributed to the different groups.

Sperm analyses and statistical analyses

At least 100 spermatozoa were analyzed per sample using Image J (National Institutes of Health, Bethesda, MD, USA). The sperm heads were circled in brightfield, and only those with attached tails were selected for analysis. The RF intensity of sperm heads exhibiting PLCζ was then quantified with the region of interest (ROI) tool in ImageJ software. The RF intensity of PLCζ in the ROI for each experiment was then normalized to the background ROI. The mean RF intensities represent the mean PLCζ levels in sperm from each male (in arbitrary units [a.u.]); the mean sperm PLCζ level between staining methods was compared. The proportion of spermatozoa exhibiting PLCζ was also assessed where appropriate, as were PLCζ localization patterns.

Data were analyzed with Prism software 5.0 (GraphPad, San Diego, CA, USA). Raw data were tested for normality with the D'Agostino & Pearson omnibus test, and statistical analysis was performed with one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. Differences were statistically significant at $P < 0.05$. Data were expressed as mean ± standard error of mean (s.e.m.), unless stated otherwise.

RESULTS

Experiment 1: AUM and visualization of PLCζ in frozen spermatozoa from fertile donors

Comparison of RF intensities of PLCζ in AUM-treated (AT or HCl) and non-AUM-treated spermatozoa from each individual allowed us to test whether the detection of PLCζ changed according to the reagent used. **Figure 1a** shows that RF intensity of PLCζ varied among the HCl-AUM, AT-AUM, non-AUM, and in-house groups. Of the 14 donors, 6 showed a significant increase in PLCζ levels in the AT- and HCl-AUM groups compared with those in the non-AUM group (all $P < 0.05$). PLCζ levels were increased in 8 of 14 samples in the HCl-AUM group compared with those in the non-AUM group (donors 1–6, 11, and 13); of these, two were significantly increased (donors 1 and 4, $P < 0.01$ and $P < 0.05$, respectively). AT was more efficient in improving RF intensity of PLCζ than HCl; of the 14 donors, 10 showed higher PLCζ levels in the AT-AUM group than the non-AUM group (donors 2–6, 8, and 10–13), of which 5 donors were significantly different (donors 3, 4, 6, 8, and 12, all $P < 0.05$). Nevertheless, of the 14 donors, HCl-AUM spermatozoa from 6 donors (donors 7–10, 12, and 14), and AT-AUM spermatozoa from 4 donors (donors 1, 7, 9, and 14), showed reduced RF intensity of PLCζ compared with the non-AUM group, and one from each group was statistically significant (donors 7 and 9, $P < 0.001$ and $P < 0.05$, respectively).

Comparison between the in-house and AUM methods showed that the former exhibited better visualization of PLCζ RF. The in-house method led to significantly higher PLCζ levels in 13 of 14 males than either AT-AUM or HCl-AUM (except donor 5; **Figure 1a**). Notably, 8 of 14 males showed significantly higher PLCζ levels in sperm stained with the in-house method than any of the AUM methods (donors 2, 4, 6, 7, 9–11, and 14, all $P < 0.05$; **Figure 1a**). Only three donors had lower PLCζ levels in spermatozoa stained with the in-house method than those from HCl-AUM or AT-AUM group, and no difference was significant (donors 1, 8, and 12; **Figure 1a**).

We also investigated the overall mean PLCζ level in each of the four groups. The overall mean levels of PLCζ in HCl- and AT-AUM groups were higher than those in the non-AUM group (**Figure 1b**), but these differences were not significant. However, overall, the in-house group exhibited a significantly higher RF intensity than the other groups (18.87 ± 2.39 a.u., $P < 0.05$).

We also used confocal microscopy to confirm our ability to visualize PLCζ. **Figure 1c** shows representative images of PLCζ

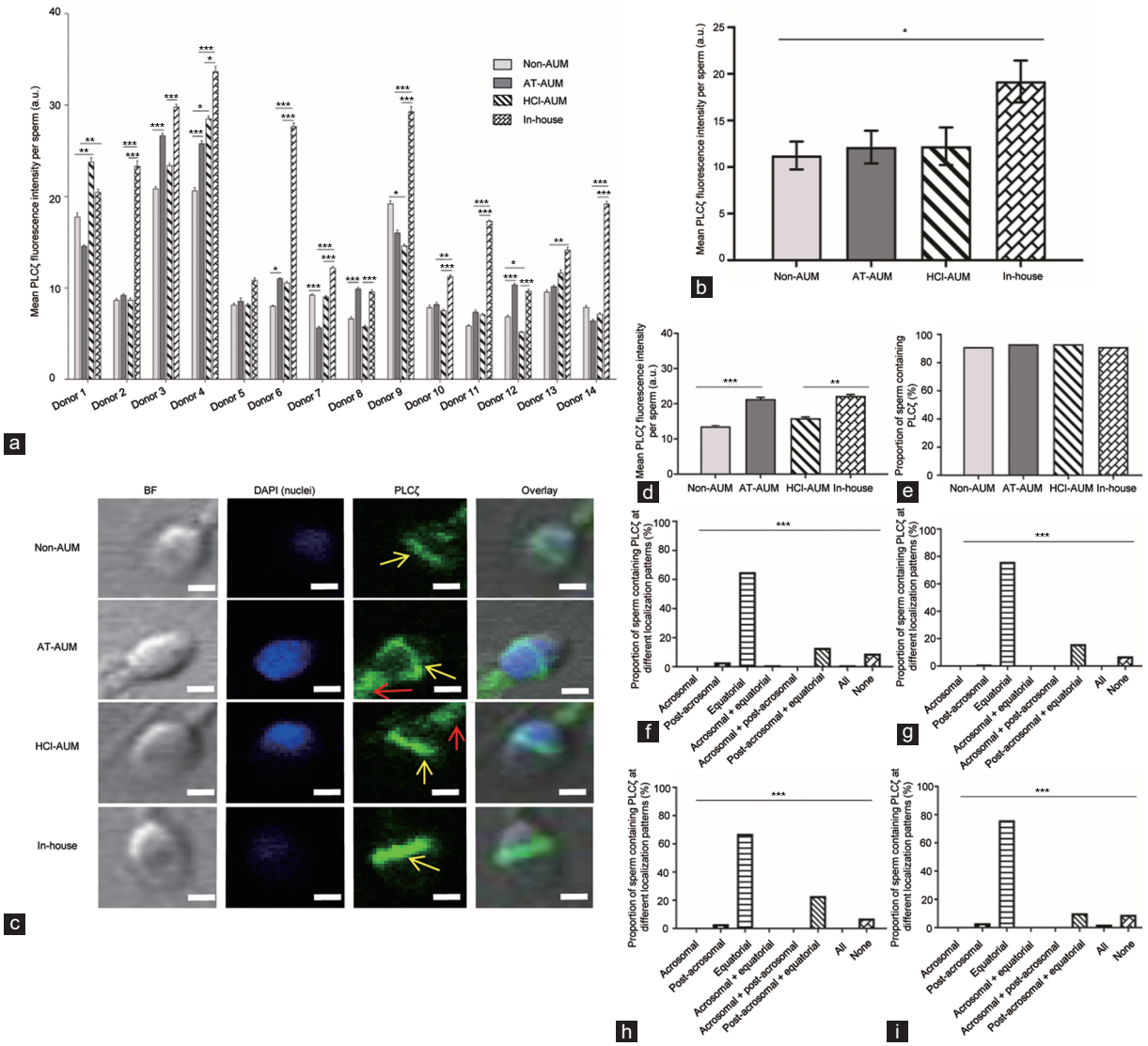

Figure 1: (**a–e**) Comparison of PLCζ visualization in frozen-thawed spermatozoa from fertile donors between in-house and AUM methods. (**a**) Mean PLCζ levels in spermatozoa from 14 fertile individual donors. RF intensity of PLCζ was compared between AUM and in-house methods, and between methods with and without AUM reagents. (**b**) Overall mean PLCζ levels in spermatozoa from a total of 14 fertile donors. Data represent mean ± s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, with one-way ANOVA and Bonferroni correction. (**c–i**) Comparison of PLCζ visualization in spermatozoa from a fertile donor (donor 3) between "in-house" and AUM methods. (**c**) Confocal microscopy visualization of PLCζ. The in-house method exhibited better RF intensity of PLCζ in the equatorial segment of the sperm head than AUM methods. Extensive RF intensity of PLCζ was observed in the midpiece of AUM-treated (AT or HCl) sperm (red arrows). Yellow arrows indicate PLCζ. Representative images for BF microscopy, DAPI, FITC-PLCζ staining, and overlay showing sperm expressing PLCζ. Scale bars = 5 μm. (**d**) Overall mean RF intensity of PLCζ in spermatozoa from donor 3. Data represent mean ± s.e.m. (**e**) Proportions of spermatozoa exhibiting PLCζ. Data represent mean values. $^{**}P < 0.01$ and $^{***}P < 0.001$, with one-way ANOVA and Bonferroni correction. (**f–i**) Comparison of PLCζ localization in spermatozoa. PLCζ localization was classified into eight patterns and compared between spermatozoa stained with (**f**) non-AUM, (**g**) AT-AUM, (**h**) HCl-AUM, and (**i**) in-house methods. Data represent mean percentages. $^{***}P < 0.001$ with one-way ANOVA and Bonferroni correction. PLCζ: phospholipase C zeta; AUM: antigen unmasking method; RF: relative fluorescence; a.u.: arbitrary units; s.e.m.: standard error of mean; ANOVA: analysis of variance; DAPI: 4'-6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; BF: bright field; AT: acidic Tyrode's solution.

Table 2: Study design of each experiment

Experiment number	*Spermatozoa*	*Group*
1	Frozen-thawed spermatozoa from fertile donors (*n*=14)	(1) In-house, (2) AT-AUM, (3) HCl-AUM, and (4) non-AUM
2	Fresh-fixed spermatozoa from infertile patients (*n*=12)	(1) In-house, (2) AT-AUM, (3) HCl-AUM, and (4) non-AUM
3	Fresh-fixed spermatozoa from infertile patients (*n*=3)	(1) AT-in-house, (2) HCl-in-house, and (3) in-house
4	Specificity testing of binding between PLCζ antigen and antibody, frozen spermatozoa from a fertile donor	(1) In-house, (2) AT-AUM, (3) HCl-AUM, and (4) non-AUM

AUM: antigen unmasking; AT: acidic Tyrode's solution; HCl: hydrogen chloride; PLCζ: phospholipase C zeta

visualized in frozen-thawed spermatozoa from donor 3, whose spermatozoa had shown increased RF intensity of PLCζ in the AT-AUM group over that in the non-AUM group (donor 3, $P < 0.001$; **Figure 1a**). Confocal microscopy showed that the RF intensity was stronger in the midpiece of AUM-treated sperm than that in the non-AUM-treated and in-house-stained sperm (**Figure 1c**, red arrow).

The overall comparison results of mean sperm PLCζ levels after staining with different methods were similar when using confocal microscopy, except that confocal microscopy provided better resolution and yielded stronger RF intensity (**Figure 1d**). In brief, AT-AUM significantly increased PLCζ levels in sperm compared with levels in the non-AUM group. HCl-AUM also increased sperm PLCζ levels, but this was not statistically significant. The in-house method remained the most efficient method compared with the other methods, with a significant difference between the in-house and HCl-AUM methods (in-house group: 22.23 ± 0.39 a.u., *vs* HCl-AUM group: 15.91 ± 0.34 a.u., $P < 0.01$).

We also analyzed the proportion of spermatozoa exhibiting PLCζ in their heads and PLCζ localization patterns under confocal microscopy, in the sample from donor 3. Proportions of spermatozoa expressing PLCζ were similar between different staining methods **(Figure 1e)**. The distribution of different PLCζ localization patterns in sperm heads were also similar, with dominant equatorial PLCζ localization identified in all staining methods **(Figure 1f–1i)**.

Experiment 2: The ability of the in-house method to determine PLCζ levels in infertile males

As the aim of the AUM is to improve poor sperm PLCζ visualization, we also examined this method on spermatozoa from 12 infertile males. These patients attended Oxford Fertility seeking treatment, and the in-house assay showed that they had significantly reduced sperm PLCζ levels compared to fertile controls. In the present study, the overall mean PLCζ level in AT-AUM and HCl-AUM spermatozoa from the 12 patients was slightly higher than that in the non-AUM group, but this was not significant (**Figure 2a**). However, the in-house method showed a significantly higher RF intensity of PLCζ than both the HCl-AUM and AT-AUM methods (**Figure 2a**).

The ability of the AUM (AT and HCl) and in-house methods to increase RF intensity was also compared with that of spermatozoa from nine individual patients (**Figure 2b**). AUM did not achieve consistent visualization of sperm PLCζ in infertile males. Specifically, of the 12 patients, 9 showed increased PLCζ levels after AT-AUM or HCl-AUM treatment compared with the non-AUM group (patients 2, and 4–11; **Figure 2b**); however, only two were significant (patient 6 and 10, both $P < 0.001$). Meanwhile, AT- and HCl-AUM also yielded reduced sperm PLCζ levels in 7 out of 12 patients, compared with the non-AUM method; two of these were statistically significant (patient 4 and 12, $P < 0.01$ and $P < 0.05$, respectively; **Figure 2b**).

Compared with the inconsistencies in PLCζ levels induced by the AUM across individual patients, the in-house method achieved good and consistent visualization; 10 of the 12 infertile males exhibited significantly higher RF intensity of PLCζ after in-house staining than with the other staining methods (patients 1, 3, 4, and 6–12, all $P < 0.05$; **Figure 2b**). The other two patients showed lower sperm RF intensity of PLCζ after in-house staining than AT-AUM or HCl-AUM staining; this difference was not statistically significant (patients 2 and 5; **Figure 2b**).

Experiment 3: AUM requirements for the in-house method

Aside from AUM treatments with acidic solutions, there were other differences between the two protocols, such as BSA concentration

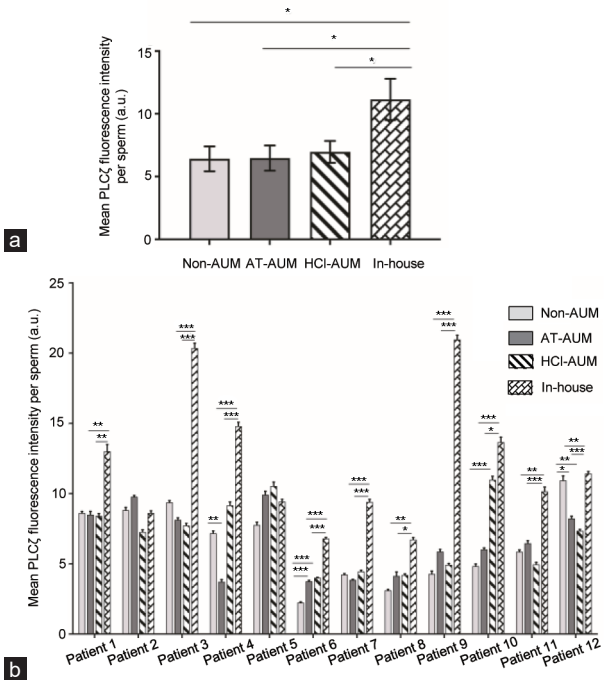

Figure 2: Comparison of PLCζ visualization in fresh-fixed spermatozoa from infertile males between "in-house" and AUM methods (n=12). (**a**) Overall mean levels of PLCζ in spermatozoa from 12 infertile males. (**b**) Mean levels of PLCζ in spermatozoa from 12 infertile males. Data represent mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with one-way ANOVA and Bonferroni correction. PLCζ: phospholipase C zeta; AUM: antigen unmasking method; RF: relative fluorescence; a.u.: arbitrary units; s.e.m.: standard error of mean; ANOVA: analysis of variance; DAPI: 4'-6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; BF: bright field; AT: acidic Tyrode's solution.

(**Table 1**). To investigate whether the RF intensity of sperm PLCζ was improved by acidic antigen unmasking reagents (AT and HCl), we applied these reagents to sperm while following the in-house protocol.

Following the in-house protocol, the overall mean sperm RF intensity of PLCζ in three infertile males was not significantly increased after HCl or AT treatment (**Figure 3a**). By contrast, the in-house method without AT/HCl led to the highest overall RF intensity of PLCζ, although this difference was not significant (P=0.15; **Figure 3a**). Of the three males, two showed significantly reduced sperm PLCζ levels after exposure to AT or HCl (patients 2 and 3, $P < 0.05$ and $P < 0.001$, respectively; **Figure 3b**).

Experiment 4: The in-house method and non-specific binding

Because the in-house method generally showed higher sperm RF intensity of PLCζ than AUM, we investigated antibody binding specificity to determine whether the high RF intensity of PLCζ was due to unspecific binding.[25] Samples of non-AUM and AT-AUM spermatozoa both exhibited visible fluorescence after peptide blocking, compared with the in-house method (**Figure 4** and **Supplementary Figure 1**). Peptide blocking was performed on spermatozoa from a fertile donor who had previously exhibited higher RF intensity of PLCζ with the in-house method than with AT-AUM and non-AUM staining (donor 9, $P < 0.001$; **Figure 1a**), indicating that the RF intensity of PLCζ was specific when tested by the in-house method. In addition, strong RF intensity in the sperm midpiece after peptide blocking was observed (**Figure 4**).

DISCUSSION

This study compared staining outcomes between our established in-house method for detecting sperm PLCζ and a recently published

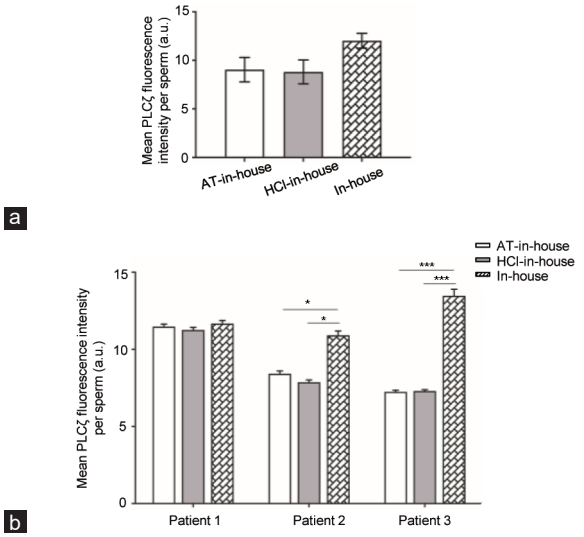

Figure 3: Investigation of the applicability of AUM reagents with the in-house method. (**a**) Overall mean RF intensity of PLCζ in spermatozoa from infertile males (n=3). (**b**) Mean RF intensity of PLCζ in fresh-fixed spermatozoa from individual patients. $^{*}P < 0.05$ and $^{***}P < 0.001$ with one-way ANOVA and Bonferroni correction. Data represent mean ± s.e.m. PLCζ: phospholipase C zeta; AUM: antigen unmasking method; RF: relative fluorescence; a.u.: arbitrary units; s.e.m.: standard error of mean; ANOVA: analysis of variance; DAPI: 4′-6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; BF: bright field; AT: acidic Tyrode's solution.

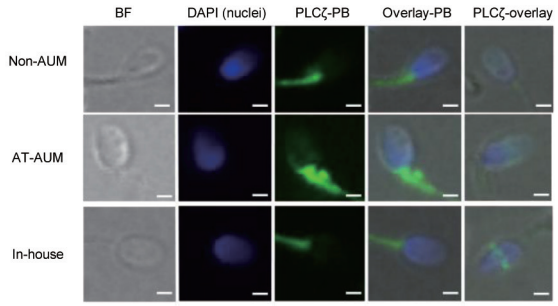

Figure 4: Comparison of specific antigen–antibody binding between "in-house" and AUM methods, using PLCζ peptides for PB. Representative images after PB (fertile donor 9) in BF microscopy, DAPI staining, FITC-PLCζ staining and overlay-PB, and PLCζ staining before PB. The in-house method-stained spermatozoa lacked PLCζ staining after PB, while AT-treated spermatozoa exhibited RF intensity after PB. Scale bars = 5 μm. PLCζ: phospholipase C zeta; AUM: antigen unmasking method; RF: relative fluorescence; DAPI: 4′-6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; BF: bright field; AT: acidic Tyrode's solution. PB: peptide blocking.

AUM.[25] Results showed that the in-house method stained sperm antigen targets with good specificity and consistently exhibited the best overall visualization, indicating that AUM is not a superior method for PLCζ immunoanalysis nor the most viable tool for clinical assessments.

PLCζ is the key factor responsible for initiating oocyte activation by inducing Ca^{2+} oscillations. Thus, the quantitative analysis of PLCζ expression can help us to investigate the oocyte activating capability of spermatozoa from infertile males and determine their chances of fertilization.[29] To estimate the levels of PLCζ in patients, it is critical to ensure good visualization after staining. We found that staining spermatozoa with our established in-house method was effective and is consistent with the results of previous studies conducted by our research group.[14–16,18,21,22] Other research groups have also used immunofluorescence to study human sperm PLCζ expression levels using the same antibody and have also observed good antibody–antigen affinity in sperm from fertile controls, without the need for antigen retrieval (*i.e.*, without reversing epitope masking).[13,19,20,30–32]

Kashir *et al.*[25] recently suggested that poor specificity between the antibody and masked PLCζ antigens resulted in poor visualization. These authors further speculated that such poor affinity may lead to the miscalculation of PLCζ levels in sperm from males attending IVF clinics, thereby impairing subsequent clinical decisions. It is worth mentioning that Kashir *et al.*[25] used fresh-fixed fertile human sperm in the AUM study, while our study used both frozen and fresh-fixed sperm. The two preservation methods may cause variable RF intensities of PLCζ; frozen-thawed sperm can exhibit lower RF intensity than fresh-fixed sperm.[22,32]

The aim of our assay is to provide useful and accurate information to assist clinical decision-making for AOA. The clinical outcomes of the 12 infertile patients recruited by the current study showed that one patient (patient 6) opted to undergo AOA treatment and achieved a successful live birth. Interestingly, patient 6 exhibited significantly higher PLCζ levels in spermatozoa from in-house group than those in the AUM groups. The good RF intensity in sperm stained with the in-house method indicates that this assay is optimal for assessing PLCζ expression. Furthermore, the successful live birth achieved by patient 6 indicates that the AUM may not be necessary when deciding whether to use AOA with particular patients. Indeed, our in-house assay has been proven accurate in identifying candidates who may need AOA treatment.[27]

In addition, the high immunoreactivity in spermatozoa from fertile donors observed in the current study indicates that the low sperm PLCζ levels observed in infertile males are not due to an ineffective immunofluorescence technique. Dysfunctional regulatory genes or transcriptional/translational factors can be possible reasons for impaired protein expression. Genetic abnormalities in PLCζ exons can influence the proportion of spermatozoa expressing PLCζ,[33] protein enzymatic activity,[1,34] or stability.[35] The underlying mechanisms responsible for impairing PLCζ protein levels in spermatozoa from infertile males remain unknown and need to be further investigated.

Apart from utilizing AT and HCl reagents for AUM, we also attempted to heat the spermatozoa to retrieve antigens, as suggested previously.[25] However, it is difficult to replicate this method because the specific heating temperature was not mentioned in the original publication. We therefore heated sperm in sodium citrate at 95°C for 7 min, because 95°C is commonly used in antigen retrieval method. However, results from these experiments were not conclusive.

However, it is unlikely that aldehyde-induced cross-linkage affects PLCζ staining, because cross-linking, in theory, takes place randomly, and because the effects of cross-linking are likely to be limited to alterations of a few protein epitopes or subtle three-dimensional conformational changes.[36] Notably, the current study used an antibody produced by the Coward Laboratory, which was raised against human PLCζ amino acid sequences to bind two epitopes of the PLCζ protein (the EF and C2 domains) and provide good visualization, suggesting that it is unlikely that aldehyde fixation masks PLCζ antigens in spermatozoa. Interestingly, formaldehyde-fixed spermatozoa were incubated with HCl or AT in experiment 3 using the in-house protocol, but this did not improve RF intensity of PLCζ. This indicates that these acidic reagents (which are recommended for AUM) are not effective in breaking down cross-linkages, or they are not the key factors that increase RF intensity of PLCζ.

The immunofluorescence analysis of PLCζ is based on data generated from signals emitted by a fluorescent-tagged antibody that binds to the target antigen, so specific binding between the antigen and antibody is of the utmost importance. The peptides contained amino acid sequences corresponding to PLCζ sequences, so they bound to the anti-human PLCζ antibody and hindered any further antibody binding with the PLCζ proteins in the test samples. The detection of fluorescence signals from AUM-treated sperm heads after peptide blocking indicates that some of the observed signals after AUM reflect non-specific binding, and this was not considered by Kashir *et al.*[25] The antibodies used in the current study and the AUM study[25] were both polyclonal; using monoclonal antibodies would further improve binding specificity.

The reliability and consistency of the immunoanalysis results also need to be considered. AUM staining on frozen/fresh-fixed spermatozoa from fertile/infertile males all showed variable results with the different AUM reagents used. Inconsistent responses to AUM reagents will cause difficulty in clinical settings when deciding which acid reagent to use and make the assay more complicated to perform. We also investigated the feasibility of incorporating AUM treatment into our in-house assay, but it failed to increase sperm RF intensity of PLCζ in either fertile and infertile males. This indicates that acidic treatments may not be the main reason for the increased RF intensity of PLCζ observed in the AUM study. The precise mechanisms underlying these observations now need to be investigated. Immunoblotting or fluorescent cell sorting may help provide additional information. The immunofluorescence staining of PLCζ protein in spermatozoa is an attractive method with which to quantify PLCζ protein levels. This is because this method does not require special equipment, which is easier for clinical diagnostic purposes, and that the protocol of immunostaining is simple and easy to follow.

In conclusion, this study compared immunofluorescence staining between our established in-house method and AUM for visualizing PLCζ levels in human sperm. These results demonstrated the good visualization and reliability of our in-house method compared with AUM and supports the continued use of our in-house assay for clinical screening.

AUTHOR CONTRIBUTIONS

XM, CJ and KC participated in study design. CR, GM, and TC facilitated patient recruitment. XM and CJ participated in data analysis. XM, PM and CJ drafted the manuscript. XM, CJ, PM, CR, GM, TC and KC revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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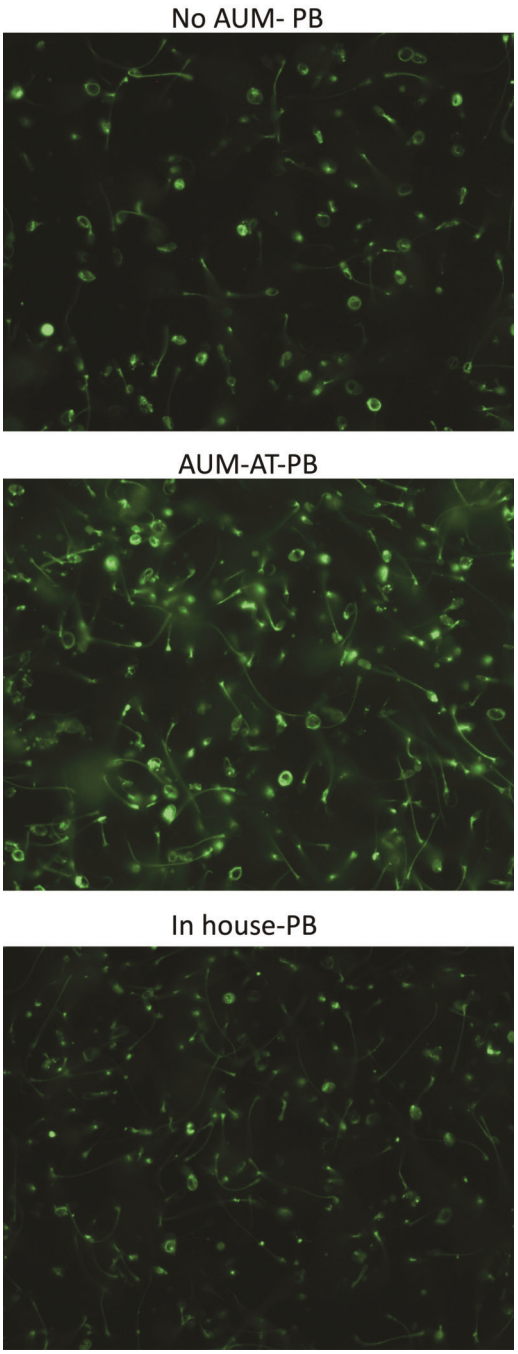

Supplementary Figure 1: Images of spermatozoa stained with non-AUM, AT, and the in-house methods after peptide blocking. Images of spermatozoa from fertile donor 9 were taken at x40. AT: acidic Tyrode's solution; AUM: antigen unmasking; PB: peptide blocking.