Supporting Information

A dual-site controlled pH probe revealing the pH in sperm cytoplasm and screening for high quality sperm

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Experimental Procedures

Subjects and controls

This study was approved by the Ethics Committees at West China Second University Hospital of Sichuan University. Informed consent was obtained from every participant. The inclusion and

exclusion criteria used in this study were selected according to previously published reports and the guidelines of the World Health Organization (WHO)[1]. Control samples of semen were collected from healthy donors whose fertility had been previously confirmed and who had a sperm count of >40 million/mL, a total motility >80%, and a progressive motility >60%. Asthenozoospermia samples of semen were collected from the male infertility clinic from patients with A+B <32% according to CASA analysis.

pH probe transfection and fluorescence detection

Briefly, 1¬2 mL of semen was washed three times with 2¬3 mL of PBS. The semen samples were then placed in 15 mL centrifuge tubes and centrifuged at 520 × g for 10 mins. Then, PBS was used to resuspend the pellet to obtain an appropriate concentration of sperm. A previously developed gold nanometer pH probe, AuNP, was added to sperm and incubated for 10 min at 25℃. The sample was then washed with PBS to remove the pH probe. A NEST 3.5-mm glass-bottom dish coated with poly-lysine was prepared in advance. Then, 3 μL of the prepared co-stained sperm was placed on the glass-bottom dishes to obtain images using laser confocal microscope (Olympus Micro).

Standard curve of AuNP pH probe

To further explore the applicability of AuNP probe in sperm, sperm were treated with each "nano-submarine" and the H+/K+ ionophore nigericin (which was applied to homogenize the endocellular pH with that of the surrounding medium), All the sperm images were collected at 500−550 nm for the fluorescein unit (the green channel, λex 488 nm), 570−650 nm for the rhodamine unit (the red channel, λex 552 nm). and then a standard intracellular pH calibration curve for each "nano-submarine" was established.

Immunofluorescence

The sperm samples were fixed onto slides using 4% paraformaldehyde for 10 min, followed by washing with PBS. The slides were permeabilised with 0.3% Triton X-100 and blocked with 5% BSA in PBS. The slides were then sequentially incubated with the corresponding primary antibodies (anti-QRICH2 (1:50) (HPA052219; Sigma-Aldrich) anti-α-tubulin (1:2000) (T7451; Sigma-Aldrich), and anti-Tyr (1:500) (Thermo Fisher)) overnight at 4°C. On the following day, the slides were washed with PBS and incubated with Alexa Fluor 488 (1:500) (A21206; Thermo Fisher) or Alexa Fluor 594 (1:500) (A11005; Thermo Fisher)-labelled secondary antibodies for 1 h at room temperature, followed by counterstaining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) to label the nuclei. Images were obtained using a laser scanning confocal microscope (Olympus). Some of the sperm samples (asthenozoospermia and control) were treated with NNC 55-0396 or progesterone at different conditions prior to fixation.

Image-based flow cytometric data acquisition and analysis

Using a Flow Sight flow cytometer (FS) fitted with a ×20 microscope objective (numerical aperture of 0.9) with an imaging rate up to 2000 events per s. The sheath fluid was PBS (without Ca2+ or Mg2+). The flow-core diameter and speed was 10 μm and 66mm per s, respectively. Raw image data were acquired using INSPIRE® software. To produce the highest resolution, the camera setting was at 1.0 μm per pixel of the charged coupled device. In INSPIRE® FS data acquisition software, two bright-field channels were collected (channels 1 and 9), one FZ3 image (channel 2), one PI image (channel 5), one side scatter (SSC; channel 6), one H33342 (channel 7), and one PNA-AF647 image (channel 11), with a minimum of 10,000 spermatozoa collected. The following lasers and power settings were used: 405 nm (to excite H33342): 10 mW; 488 nm (to

excite FZ3): 60 mW; 561 nm (to excite PI): 40 mW, 642 nm (to excite PNA-AF647): 25 mW; and 785 nM SSC laser: 10 mW.

Sperm viability

After pH probe incubation, the sperm were mixed with eosin staining solution (Puhualife, China) equal volume and stained for 5 minutes. Then the sperm was smeared to slides and observed under the microscope. were counted, and the percentage of unstained sperm was calculated in two hundrads sperm.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). All statistical analysis was performed using GraphPad Prism software (version 8.00, CA, USA). For the comparison between two groups, a two-tailed Student's t-test was used. For the comparison of more than two groups, statistical comparisons were performed using one-way analysis of variance (ANOVA). Significant differences between or among groups are indicated by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, respectively.

Supplementary Figures

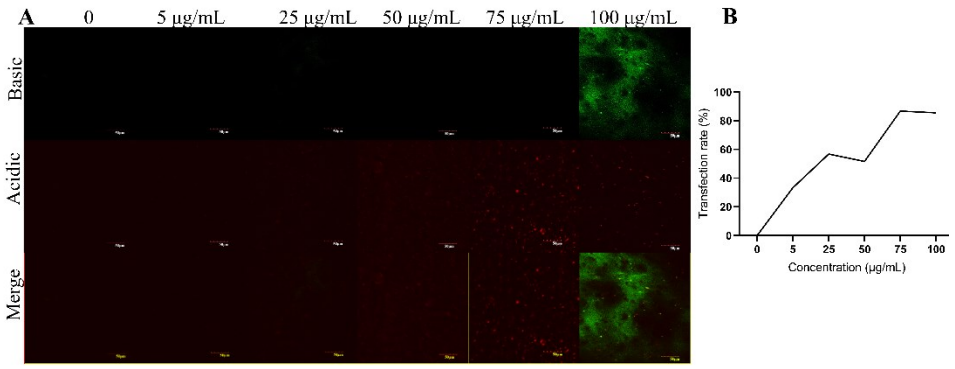

Fig. S1 The fluorescence images(A) and statistic(B) of different concentrations of pH probes in sperm. Scale bars: 50 μm

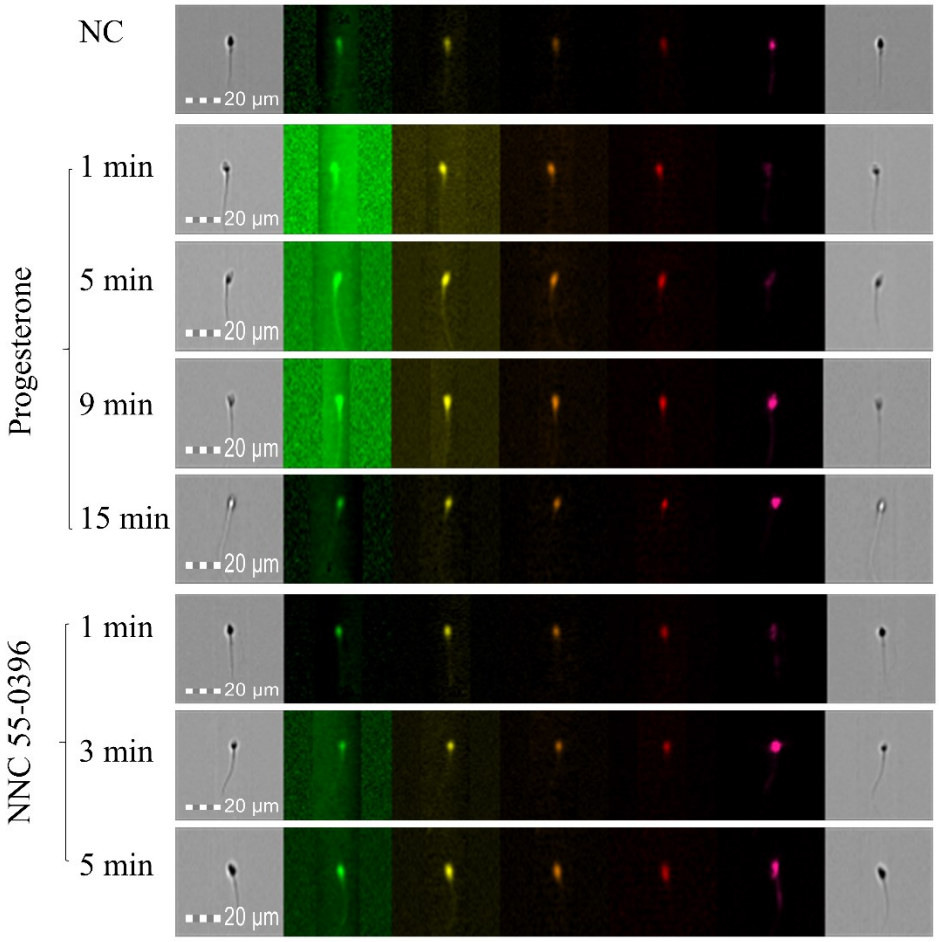

Fig. S2 Imaging results of single sperm captured by IBFC under different processing conditions after adding AuNPs. Scale bars: 20 μm

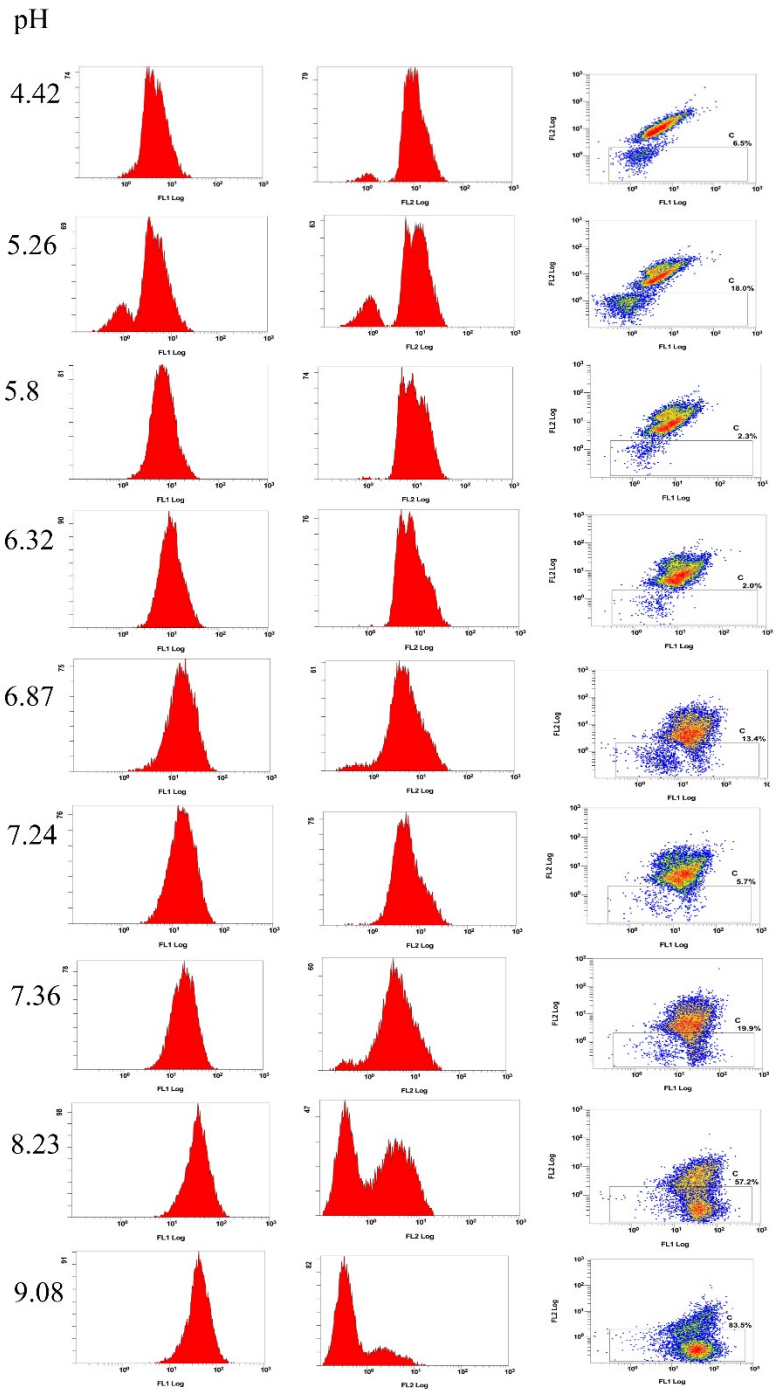

Figure S3 Flow cytometry images of sperm at different pH with AuNP (75 μg/mL) after treatment with nigericin.

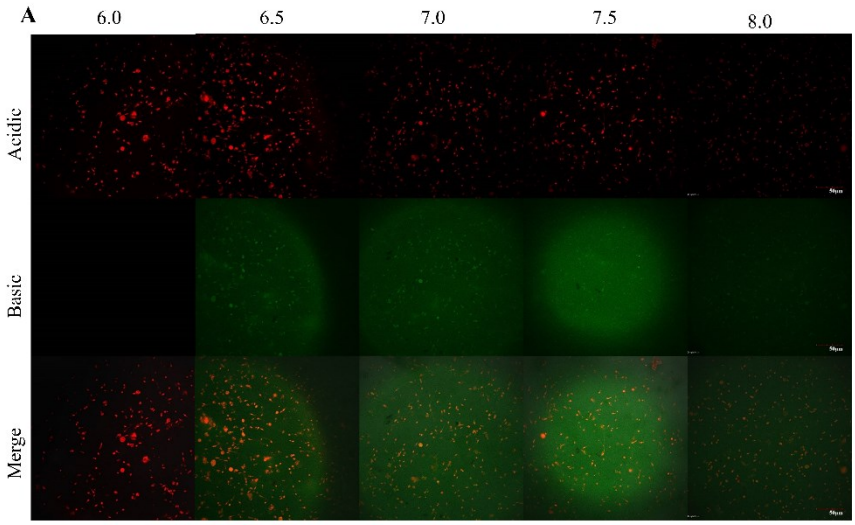

Fig. S4 The fluorescence images of AuNPs(75 μg/mL) in sperm at pH from 6.0 to 8.0. Scale bars: 50 μm

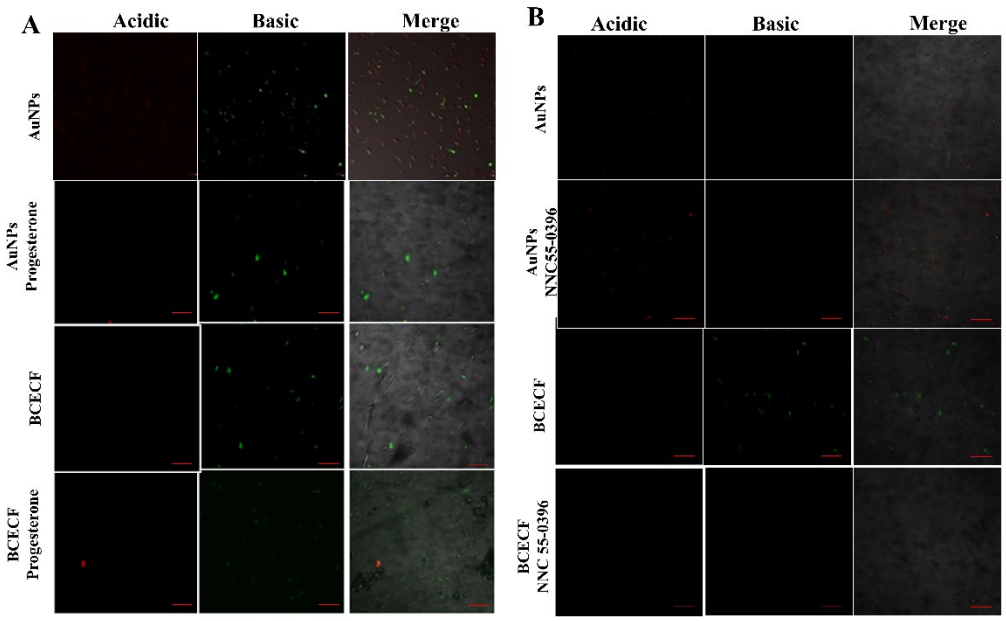

Figure S5 Treated sperm with progesterone (A) and NNC 55-0396 (B), compare the staining characteristics of BCECF and AuNPs. Scale bars: 50 μm

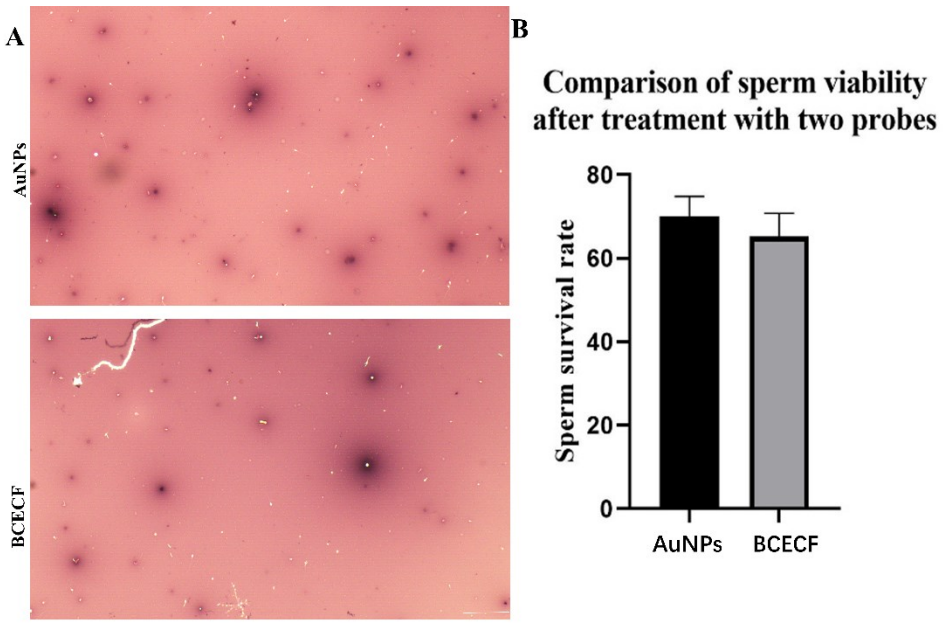

Fig. S6 Comparison of changes in sperm viability rate after the treatment of BCECF and AuNP

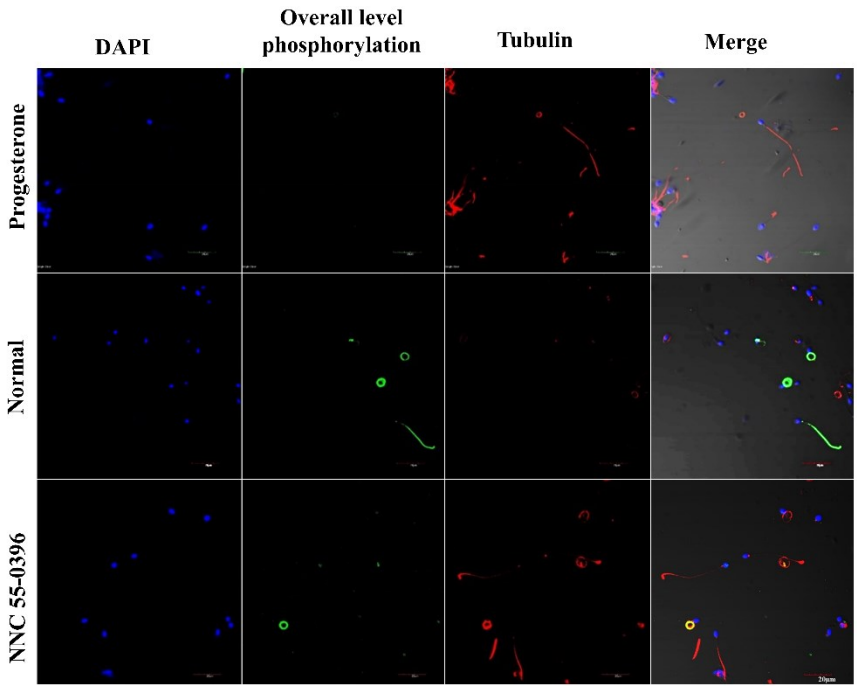

Fig. S7 The overall level phosphorylation of sperm at different conditions. Scale bars: 50 μm

Supplementary Tables

Table S1 The comparison of TG AuNPs, AuNCs and AuNPs .

	pH resolution	responsive pH range	reusability
TG AuNPs	<10%	5.0-11.0	6.0-10.0
AuNCs	Δ pH < 0.35	5.5-7.5	6.05-6.40
AuNPs		4.5-8.0	6.0-8.0

Table S2 Comparison of AuNPs applied between hela cell and sperm.

	Hela cell	Sperm
The time of incubation	30 min	30 min
The concentration of AuNPs	50 μg/mL	75 μg/mL
The temperature of incubation	37 ℃	20-25 ℃
The formula of linear transformation	Linear fit	Logarithmic transformation
The pH range of linearity	6.0-8.0	5.2-8.2

Table S3 The statistics of Flow cytometry of sperm at different pH with AuNP (75 μg/mL) after treatment with nigericin.

pH	X is Green	Y is Red	G/R	Number	Total%	Gate%	All-Number
4.42	1.88	1.02	1.843137	757	5.05	6.53	11598
5.26	1.03	0.876	1.175799	2206	14.71	18.04	12230
5.8	2.32	0.99	2.343434	292	1.95	2.34	12458
6.32	4.1	0.838	4.892601	244	1.63	1.95	12507
6.87	14.7	1.06	13.86792	1687	11.25	13.41	12583
7.24	16	1.27	12.59843	729	4.86	5.69	12820
7.36	23.6	1.08	21.85185	2491	16.61	19.9	12519
8.23	40.4	0.627	64.43381	7170	47.8	57.2	12536
9.08	45.2	0.484	93.38843	10045	66.97	83.47	12034

Table S4 Comparison of AuNPs and BCECF-AM applied in sperm.

	AuNPs	BCECF-AM
The viability of sperm	70%	65%
The concentration of incubation	75 μg/mL	2150 μg/mL
Λex	450nm (Fluorescein)	488nm
	550nm (Rhodamine)	
Λem	510nm (Fluorescein)	535nm
	580nm (Rhodamine)	

The color of fluorescence	Red/Green	Green
The pH range	5.2-8.2	6.2-9.5

References

1. Y. Shen, F. Zhang, F. Li, X. Jiang, Y. Yang, X. Li, W. Li, X. Wang, J. Cheng and M. J. N. c. Liu, 2019, **10**, 1-15.