Supporting Information

Jin et al. 10.1073/pnas.1018202108

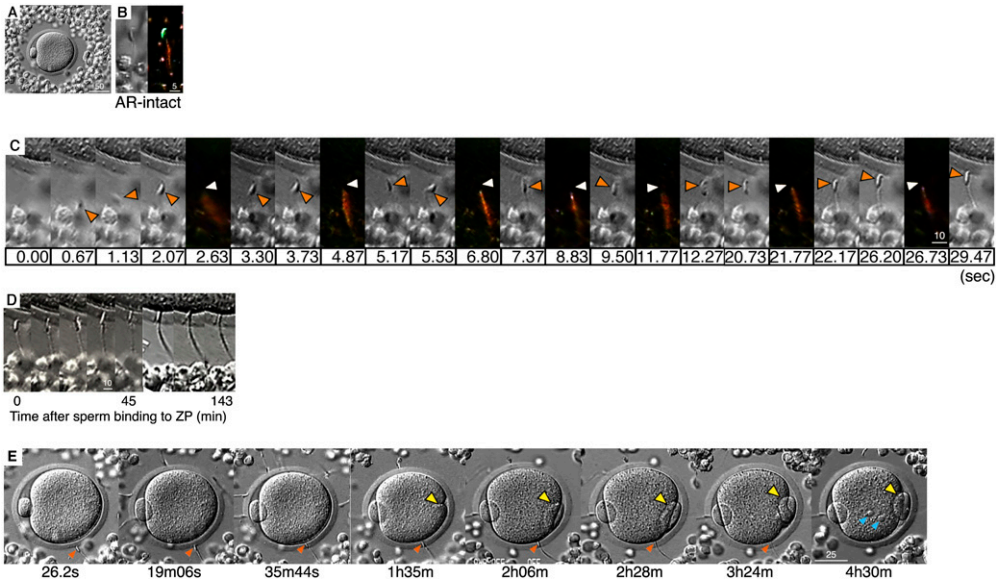


Fig. S1. A case of fertilization by a spermatozoon that has undergone the AR before its attachment to the ZP. (*A*) A low-magnification view of a cumulus-enclosed oocyte. (Scale bar, 50 μm.) (*B*) An acrosome-intact spermatozoon viewed with DIC (*Left*) and fluorescent (*Right*) microscopy. (Scale bar, 5 μm.) (*C*) Tracking the fertilizing sperm head (orange and white arrowheads) during its approach to the ZP until incorporation into the egg cytoplasm. (*D*) Dark field images show presence of mitochondrial Ds-Red2 (red), but absence of acrosomal EGFP. (Scale bar, 10 μm.) (*E*) A series of photographs shows representative processes of fertilization. Time after the appearance of the spermatozoon at the zona periphery is indicated. The spermatozoon made contact with the ZP at 26.2 s and fused with oolemma at about 35 min 44 s. Cyan arrowheads, pronuclei; yellow arrowheads, second polar body. (Scale bar, 25 μm.)

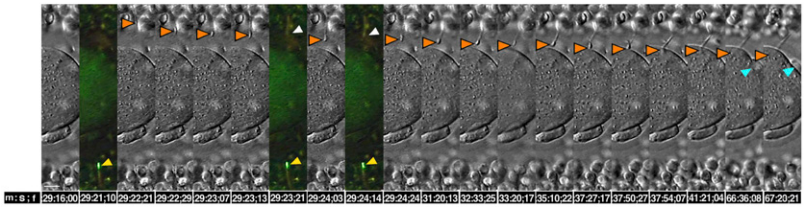


Fig. S2. A case of fertilization by a spermatozoon that has undergone the AR before its attachment to the ZP. An acrosome-reacted (absence of EGFP) spermatozoon (white arrowheads) attached to the ZP at 29 min 24.8 s after insemination. This spermatozoon was incorporated into ooplasm (orange arrowheads) at about 37 min 54 s, thereafter the second polar body formed (cyan arrowheads). Another acrosome-intact spermatozoon (yellow arrowheads) arrived at the zona periphery. m, minutes; s, seconds; f, frames (30 frames = 1 s). (Scale bar, 10 μm.)

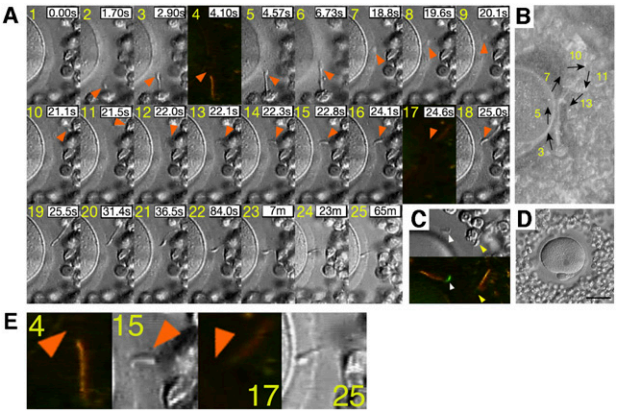


Fig. S3. A case of fertilization by a spermatozoon that has undergone the AR before its attachment to the ZP. (*A*) The head of this spermatozoon (orange arrowheads) had already lost its EGFP (acrosome-reacted) at 4.10 s (panel 4) and attached to the ZP at 22.3 s (panel 14). Time after the appearance of the spermatozoon at the zona periphery is indicated. (*B*) The trajectory of the fertilizing spermatozoon within the cumulus identified an irregular swimming behavior. Successive frames were superimposed by taking maximum gray-scale level per pixel after subtracting the background. Arrows indicate representative sperm trajectories corresponding to the panels (*A*) as indicated. (*C*) Examples of acrosome-intact (white arrowheads) and acrosome-reacted (yellow arrowheads) spermatozoa viewed under DIC (*Upper*) and fluorescent (*Lower*) microscopy. (*D*) A low-magnification view of a cumulus-enclosed unfertilized oocyte. (Scale bar, 50 μm.) (*E*) High-magnification views of a fertilizing, acrosome-reacted spermatozoon in four selected frames from *A*.

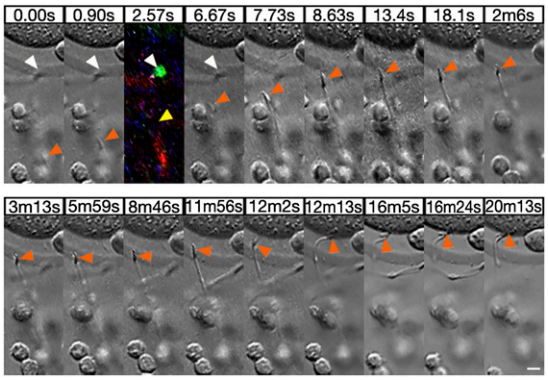


Fig. S4. Another case of fertilization by a spermatozoon that has undergone the AR before its attachment to the ZP. The fertilizing spermatozoon that came into view (0.00 s) had no acrosomal EGFP by 2.57 s (yellow arrowhead) and attached to the ZP at 8.63 s. The sperm head passed through the ZP at about 12 min, followed by fusion with the oocyte cell membrane (orange arrowheads). Another acrosome-intact ($EGFP^+$) spermatozoon (white arrowheads) was seen on the ZP.

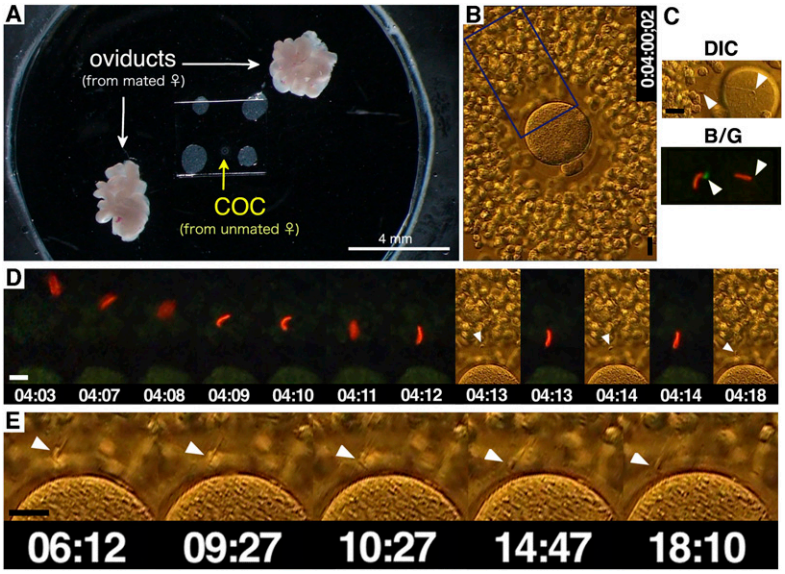


Fig. S5. In vitro fertilization by a spermatozoon released from isolated oviducts. (*A*) A low-magnification view of the in vitro fertilization setup. Oviducts that contain ejaculated spermatozoa were isolated at 12 ± 0.5 h of post-hCG injection and rinsed with HTF–BSA medium. Cumulus–oocyte complexes (COCs), if any, were removed from isolated oviducts by tearing the ampulla. These oviducts were placed next to a 3 × 3 mm^2 coverslip under which a COC from another unmated female was lying. Some of the spermatozoa that swam out of dissected oviducts reached the cumulus under the coverslip. (*B*) A photograph showing an oocyte in cumulus oophorus. The images of the rectangular area were rotated or magnified in other photographs shown in *D*. (*C*) EGFP(+) and EGFP(–) spermatozoa under a fluorescence microscope equipped with a dual bandpass (GFP/Ds-Red) filter (blue/green). Arrowheads indicate sperm heads. (*D* and *E*) A series of photographs shows passage of an acrosome-reacted spermatozoon (white arrowheads) through the cumulus and into the ZP.

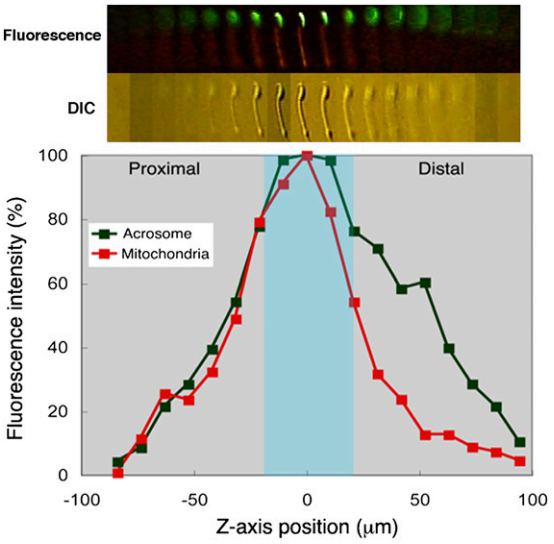
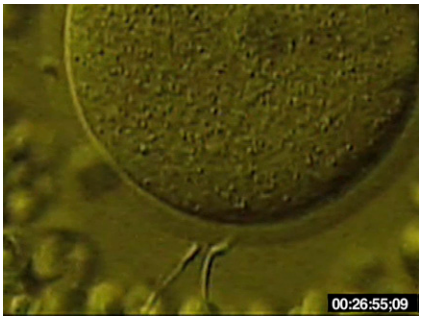


Fig. S6. Visualization of the integrity of an acrosomal vesicle in living spermatozoa. Continuous observation of mouse spermatozoon by DIC microscopy with intermittent visualization of the intact acrosomal vesicle (green) and midpiece mitochondria (red). The graph shows the relationships between the relative fluorescence (green and red) signals and the *x*-axis position at various *z*-axis positions. When sperm head was in the focal plane (cyan zone), acrosomal EGFP and mitochondrial Ds-Red2 fluorescence were visible.

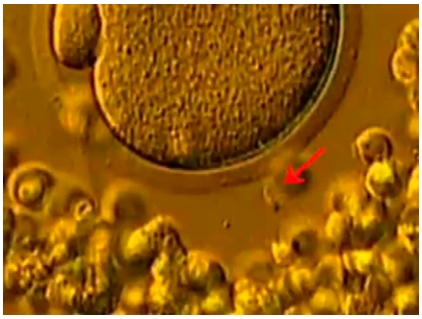
Table S1. The sites where acrosome-intact and acrosome-reacted spermatozoa advanced most closely to the ZP or reached ZP during 60–90 min of examination

Exp. no.	Total no. of sperm examined (obs. period, min)	Acrosome-intact sperm			Acrosome-reacted sperm		
		In cumulus	Near ZP	On/in ZP	In cumulus	Near ZP	On/in ZP
1	4 (60)	0	4	0	0	0	0
2	5 (60)	1	0	0	1	3	0
3	0 (90)	0	0	0	0	0	0
4	5 (60)	0	0	0	0	3	2
5	1 (60)	0	0	0	0	1	0
6	0 (90)	0	0	0	0	0	0
7	0 (90)	0	0	0	0	0	0
8	5 (60)	0	1	0	0	2	2
9	3 (60)	0	0	0	0	3	0
10	1 (76)	1	0	0	1	0	0
11	10 (60)	1	1	0	0	3	4
12	0 (90)	0	0	0	0	0	0
13	0 (90)	0	0	0	0	0	0
14	5 (60)	0	0	0	3	3	0
15	5 (60)	0	0	0	2	2	0
16	2 (60)	0	1	0	0	0	1
17	0 (90)	0	0	0	0	0	0
18	1 (60)	1	0	0	0	0	0
19	0 (90)	0	0	0	0	0	0
20	1 (60)	0	0	0	0	1	0
Total	48 (1,426)	4	7	0	7	21	9



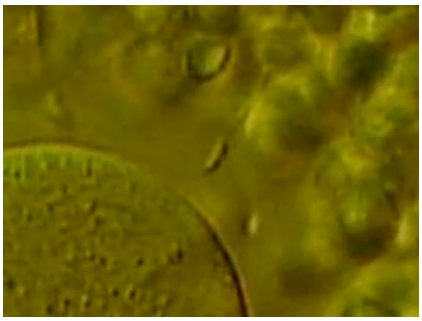
Movie S1. Real-time observation of a mouse fertilizing spermatozoon tracked through the fertilization, from interaction with the outer surface of the ZP until it developed into a pronucleus in the oocyte cytoplasm (case 1). The data presented in Fig. 3 *A–D* were obtained from this movie (QuickTime; 4.7 MB).

Movie S1



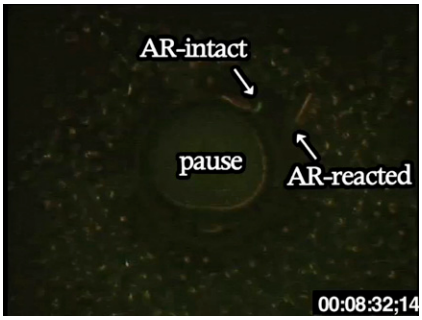
Movie S2. Representative case (case 2) where an acrosome-reacted spermatozoon bound to the ZP, thereafter this sperm fused with an oocyte. The data presented in Fig. S1 were obtained from this movie (QuickTime; 4.3 MB).

Movie S2



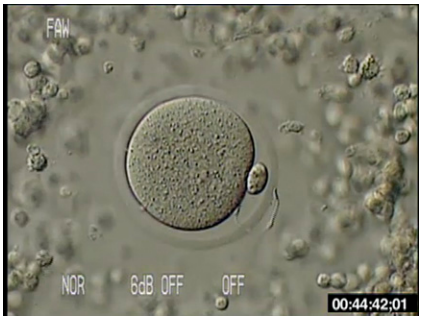
Movie S3. Representative case (case 3) where an acrosome-reacted spermatozoon bound to the ZP, thereafter this sperm fused with an oocyte. The data presented in Fig. S2 were obtained from this movie (QuickTime; 3 MB).

Movie S3



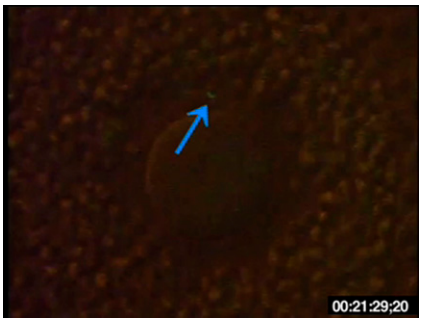
Movie S4. Representative case (case 4) where an acrosome-reacted spermatozoon bound to the ZP, thereafter this sperm fused with an oocyte. The data presented in Fig. S3 were obtained from this movie (QuickTime; 4.6 MB).

Movie S4



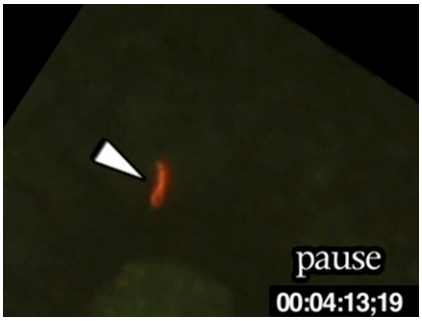
Movie S5. Representative case (case 5) where an acrosome-reacted spermatozoon bound to the ZP, thereafter this sperm fused with an oocyte. The data presented in Fig. S4 were obtained from this movie (QuickTime; 3.9 MB).

Movie S5



Movie S6. A case where an acrosome-intact spermatozoon bound to the ZP and then triggered the AR on the ZP. Thereafter, this sperm initiated ZP penetration and fused with egg plasma membrane. The data presented in Fig. 3 *E* and *F* were obtained from this movie (QuickTime; 4.4 MB).

Movie S6



Movie S7. Representative case where an oviductal sperm reached the zona periphery after the AR, thereafter bound and penetrated (partially) the ZP. The data presented in Fig. S5 were obtained from this movie (QuickTime; 3 MB).

Movie S7