

Fertilization and Sperm Chemotaxis in Ascidians

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1. Introduction

1.1. General Introduction

Prior to fertilization, spermatozoa of many animals and plants show chemotactic behavior toward eggs. Chemotactic behavior was first described in ferns (**1**), and their attractant was identified as the bimalate ion (**2**). In animals, sperm chemotaxis to the egg was first observed in the hydrozoan *Spirocodon saltatrix* (**3**) and is now widely recognized in all species from cnidarians to human (for reviews, see refs. **4–6**).

Spermatozoa of the ascidians *Ciona intestinalis* and *Ciona savignyi* were immotile or slightly motile when they were suspended in seawater, and if an unfertilized egg was placed in the sperm suspension, sperm near the egg were intensely activated and then showed chemotactic behavior toward the egg (**7–9**). Egg seawater (ESW) that is a supernatant of seawater incubated with the ascidian egg has both sperm-activating and sperm-attracting activities, indicating that the ascidian egg releases some sperm-activating and sperm-attracting factors around the egg (**9,10**). The release of the attractant from the egg seems to stop after fertilization (**9**).

1.2. Chemical Nature and Source of the Sperm Chemoattractants

Where are the sperm chemoattractants released? Fern sperm show a chemotactic response to secretions from the female reproductive structures (**1**). A sperm attractant of the sea urchin *Arbacia punctulata* is derived from the egg jelly (**11**), and the source of sperm attractant of the hydrozoan, the siphonophore, is a cupule, the extracellular structure of the egg (**12**). Therefore, the sperm attractant is released from the egg accessory organs or female gametes in these

species. On the other hand, in the ascidians *C. intestinalis* and *C. savignyi*, sperm-attracting activity does not originate from the overall egg coats, as a layer of jelly surrounds the eggs, but originate from the vegetal pole of the egg (9). This indicates that in the ascidian the eggs themselves release the chemoattractant for the sperm.

Even though sperm chemotaxis is known in many phyla of animals and plants, the chemical nature of chemoattractants has been identified in only a few species. Sperm chemoattractants in plants were identified as organic compounds with a low molecular weight: bimalate ion in the bracken fern (2) and unsaturated cyclic or linear hydrocarbon in algae (13). On the contrary, in animals, most of the known chemoattractants and candidates for them were considered to be proteins or peptides. The chemoattractant of the sea urchin *A. punctulata* called resact is a 14-amino-acid peptide (11) and that of the amphibian *Xenopus laevis* is a 21-kDa protein (14). The chemoattractants of hydrozoa and a starfish are thought to be proteins, because the sperm-attracting activity of these species was lost by protease treatment (15,16). On the other hand, chemoattractant from the eggs of the coral *Montipora digitata* is considered to be an unsaturated fatty alcohol, dodeca-2,4-diynol (17).

In the ascidians *C. intestinalis* and *C. savignyi*, ESW has both sperm-activating and sperm-attracting activities. When the sperm attractant was purified from ESW, sperm-activating and sperm-attracting activities always comigrated, suggesting that both activities are derived from a single molecule; thus, we named the attractant of *Ciona* sperm as SAAF (sperm-activating and -attracting factor) (10). Recently, we determined the chemical nature of SAAF, a novel sulfated steroid, 3,4,7,26-tetrahydroxycholestane-3,26-disulfate (18).

1.3. Analysis of Chemotactic Behavior of Spermatozoa

Precise observations of the chemotactic behavior of sperm have been performed in several species. In the hydrozoan siphonophore, the radius of curvature of the sperm trajectory reduces as the spermatozoon closes to the cupule, the source of sperm attractant (19). On the other hand, a quick turning movement is observed in the sperm of other hydrozoans, when the sperm exhibit chemotactic behavior (20,21). During the turning movement, sperm demonstrate a temporary asymmetrical flagellar beating (8,21). The ascidian sperm also show the turning movement during chemotactic behavior (7–9). The turning movement of sperm is a typical example of the behavior seen during sperm chemotaxis; therefore, it is called the “chemotactic turn.” The chemotactic turn of the hydrozoan sperm is often observed when sperm bear off the attractant (21).

As an activity assay for sperm attraction, the most commonly used method is the micropipet assay. The sample whose activity is to be examined is enclosed in the tip of a glass micropipet, the micropipet is placed in the sperm

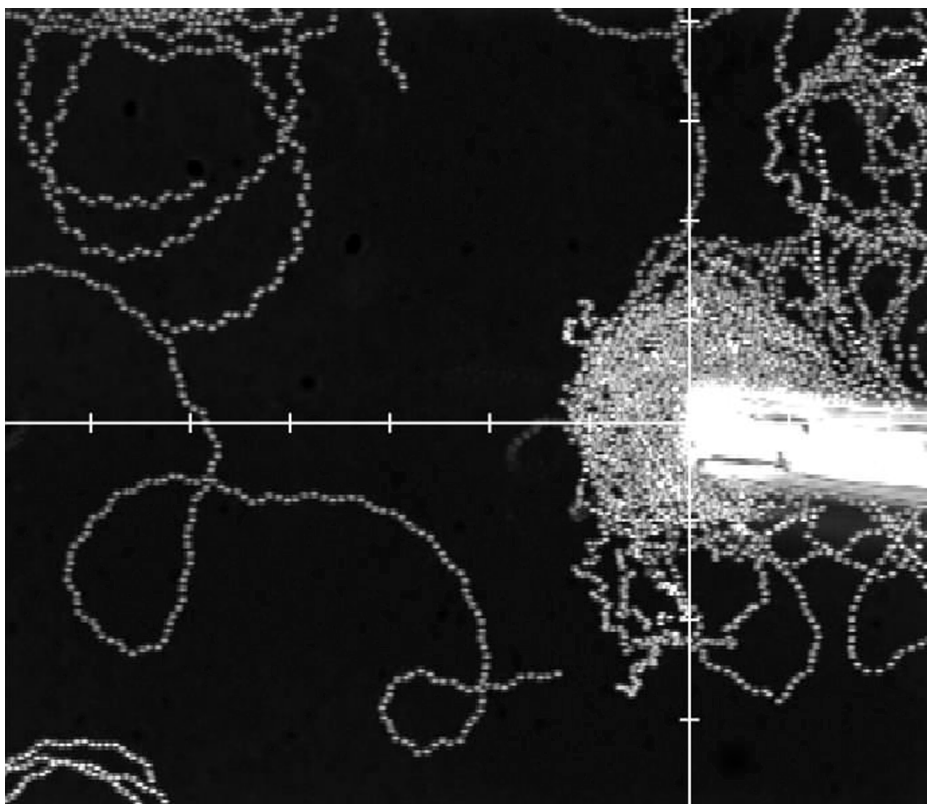


Fig. 1. Photograph of sperm trajectories near a micropipet containing ESW. The photo was integrated from 200 images taken every 20 ms. Lines show the axes for analysis. Ticks of the axes are labeled every 50 μm .

suspension, and then the sperm trajectories around the micropipet tip are observed. When a micropipet containing ESW or purified SAAF is inserted in the suspension of *Ciona* sperm, spiral trajectories of sperm toward the micropipet tip are seen, with the chemotactic turn (*see Fig. 1*).

Although molecular structures of the attractants and candidates have been proposed in several species, quantitative evaluation of sperm chemotaxis has not been well established. Furthermore, in the ascidian, it has been impossible to examine the activity of SAAF while eliminating side effects on sperm activation, because the ascidian sperm suspended in seawater has little motility. In order to distinguish the chemotactic behavior from the activation of motility in spermatozoa, spermatozoa are treated first with 1 mM theophylline for 1 min, which increases intracellular cAMP and results in sperm activation with a circular movement (*10*).

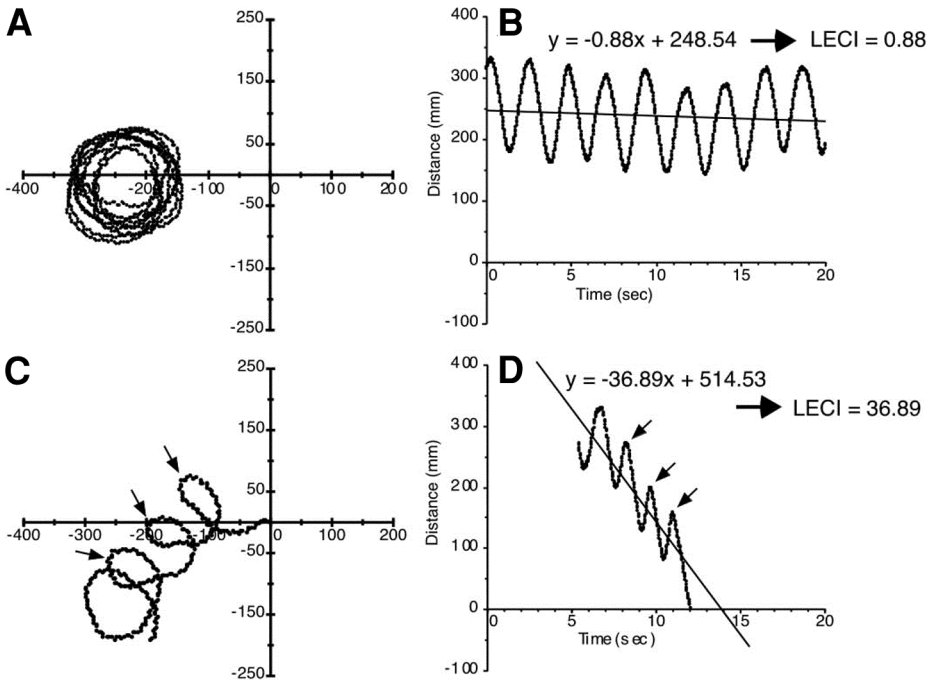


Fig. 2. Trajectories of the sperm (**A**, **C**) and plots of the distance between the sperm head and the micropipet tip against time (**B**, **D**) around the tip of micropipets containing (**A**, **B**) artificial seawater (ASW) and (**C**, **D**) ESW. The tip of the micropipet was set as the origin of coordinates (0). Arrows indicate points of the chemotactic turn. The line and formula represent the linear equation and the coefficient of time versus D , respectively. The negative value of the coefficients of the equation indicates the chemotaxis index (LECI).

My group has established a new method for quantitative evaluation of sperm chemotaxis using the linear equation chemotaxis index (LECI) (18). The LECI is a parameter that is derived from the negative value of the coefficient ($-a$) in a linear equation ($y = ax + b$) of time (abscissa in **Fig. 2**) versus the distance between the micropipet tip and the sperm head (D) (ordinate in **Fig. 2**). When the trajectories of sperm around the micropipet containing ESW were analyzed, D decreased with oscillation, although the parameter did not decrease with the addition of artificial seawater (see **Fig. 2**). The parameter LECI can represent the strength of sperm-attracting activity and will offer reliable aspects of the quantification of sperm chemotaxis.

In addition to LECI, other two parameters are useful for detailed analysis of chemotaxis in the *Ciona* sperm (18). The first is the differential quotient of D

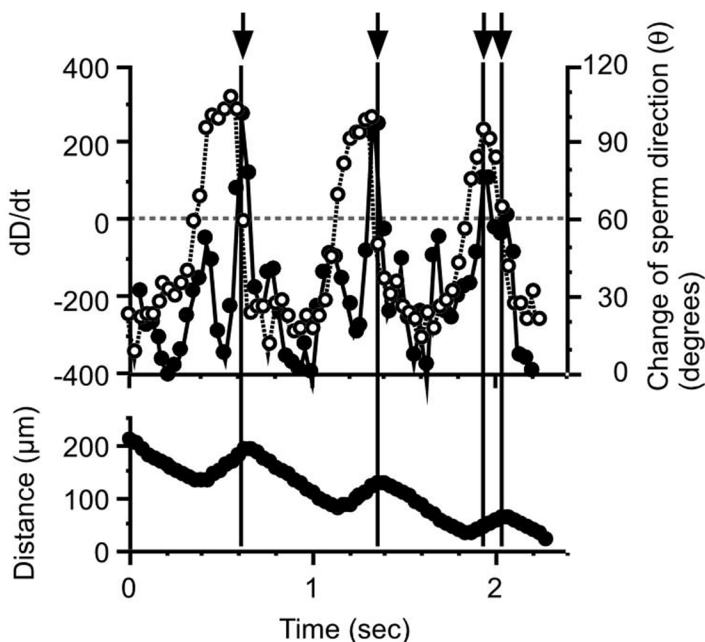


Fig. 3. Quantitative analysis of sperm chemotaxis. Changes in three parameters of a spermatozoon that showed chemotactic behavior toward purified SAAF. The points at which θ rose above 60° (i.e., the turning movement) occurred just after the peak of dD/dt (arrow) and coincided with the decrease of dD/dt .

with time (dD/dt), which represents the velocity of sperm approaching the micropipet tip. The second is the change of sperm direction (θ). The chemotactic turn can be quantitatively characterized as a quick increase in the value of θ . When *Ciona* sperm do not show chemotaxis, the sperm usually move with a constant curvature and θ is almost constant in the 0° – 30° range (see Fig. 3). However, when the ascidian sperm show chemotaxis, the sperm show a quick change in their swimming direction (the chemotactic turn) and thus the θ periodically increased to values of over 60° (see Fig. 3). Furthermore, θ always started to increase just after the peak of dD/dt and decreased coincidentally with the decrease in dD/dt (see Fig. 3). Because the positive value of dD/dt indicates that a sperm is moving away from the micropipet tip, these results suggest that the chemotactic turn occurs when sperm move away from the micropipet tip. A similar strategy was considered in the hydrozoan sperm (21). The chemotactic behavior of sperm may be controlled by the chemotactic turn that is triggered when sperm detect any decrease in the concentration of the chemoattractant.

1.4. Signaling Mechanisms of Sperm Chemotaxis

How do the sperm chemoattractants act on the movement of sperm? The requirement of extracellular Ca^{2+} for chemotaxis has been known in the bracken fern (22), hydroids (12,23), sea urchin (11), and ascidians (7–9); Ca^{2+} -chelating agents (e.g., EDTA) completely suppress chemotactic behavior of sperm. Analytical studies in the hydrozoans siphonophores showed that the diameters of the trajectories decrease upon approach of the sperm to the cupule (19). No modification of the sperm trajectories was seen in the sperms' chemotactic behavior toward the cupule in the absence of Ca^{2+} , suggesting that Ca^{2+} regulates the motility pattern of the flagellum (19). In the sea urchin *A. punctulata*, resact, the sperm-activating and sperm-attracting peptide, binds to the receptor guanylyl cyclase (24) and seems to induce an increase in $[\text{Ca}^{2+}]_i$ through cGMP and cAMP (25). The $[\text{Ca}^{2+}]_i$ elevation may be controlled by a channel like the sperm-specific cyclic nucleotide-gated and voltage-dependent Ca^{2+} channels that were recently found in the mouse (26,27) and seem to induce the asymmetry of the flagellum waveform (28–30). The same role of extracellular Ca^{2+} -induced flagellar asymmetry on induction of sperm chemotaxis has been reported in hydrozoa (19,21). On the other hand, sperm chemotaxis of the ascidians *C. intestinalis* and *C. savignyi* does not seem to require intracellular cAMP changes. When SAAF acts on sperm, it induces entry of extracellular Ca^{2+} and an increase in intracellular cAMP (10,31). This results in protein-kinase A (PKA)-dependent phosphorylation on 21-kDa and 26-kDa axonemal proteins and activation of sperm motility (32). On the other hand, the chemotactic behavior of the ascidian sperm also requires extracellular Ca^{2+} , but theophylline-activated sperm, in which cAMP increases because theophylline blocks cAMP phosphodiesterase, show the same chemotactic behavior as normal sperm (9,10). Therefore, changes in cAMP are not required for sperm chemotaxis, and the mechanism of sperm chemotaxis is different from sperm activation, even though SAAF induces both phenomena. Recently, we found that the store-operated Ca^{2+} channel looks to mediate the asymmetrical flagellar waveform of the ascidian sperm, resulting in chemotactic behavior (33).

2. Materials

1. *Ciona intestinalis*.
2. Micromanipulator.
3. Phase-contrast or dark-field microscope (see Note 1).
4. High-speed video camera system (HAS-200 and HAS-PCI; Ditect, Tokyo, Japan).
5. Image-analyzing application.
6. Micropipet puller.
7. High-performance liquid chromatograph (HPLC) equipment.

8. Rotary evaporator.
9. Freeze-dryer.
10. Centrifugal vaporizer.
11. Centrifuge.
12. Artificial seawater (ASW): 462 mM NaCl, 9 mM KCl, 11 mM CaCl₂, 48 mM MgCl₂, and 10 mM HEPES–NaOH, pH 8.2.
13. 20 mg/mL Bovine serum albumin (BSA, Fraction V).
14. 25 mM theophylline.
15. 2% agar.
16. Ethanol (HPLC grade).
17. Chloroform (HPLC grade).
18. Acetonitrile (HPLC grade).
19. Methanol (HPLC grade).
20. Sep-Pak C18 resin (Waters, Milford, MA).
21. Reversed-phase HPLC ODS column.
22. Empty column (25 mm diameter × 150 mm).
23. Glass slide.
24. Glass capillaries with inner glass fiber (outer diameter = 1 mm) (Narisige GD-1).
25. Pasteur pipet.
26. Scissors.
27. Plastic dish (diameter = 3.5 cm).

3. Methods

The following methods outline (1) the collection of sperm and eggs, (2) the purification of SAAF, and (3) the assay for sperm chemotaxis.

3.1. Collection of Gametes

1. Keep collected *C. intestinalis* in aquaria with continuously flowing seawater. To prevent spontaneous spawning, light the animals continuously until use.
2. Remove the tunic and open the body with scissors. Collect eggs and semen from the oviduct and vas deferens respectively, with Pasteur pipets. Keep eggs and semen at 16–18°C and 4°C, respectively.

3.2. Purification of SAAF

The purification schema is shown in **Fig. 4**.

1. Wash the collected eggs once with ASW and suspend them in 40 vol of ASW. Incubate the egg suspension for 14–20 h at 4°C.
2. Centrifuge the egg suspension at 1.6×10^3g for 15 min and obtain the supernatant. Centrifuge the supernatant at 2.2×10^4g for 30 min at 4°C. The obtained supernatant is the ESW.
3. Lyophilize the ESW.
4. Add 1/10 vol of absolute ethanol to the residues of the ESW and vortex the mixture. Centrifuge at 2.2×10^4g for 15 min at 4°C and transfer the supernatant to a

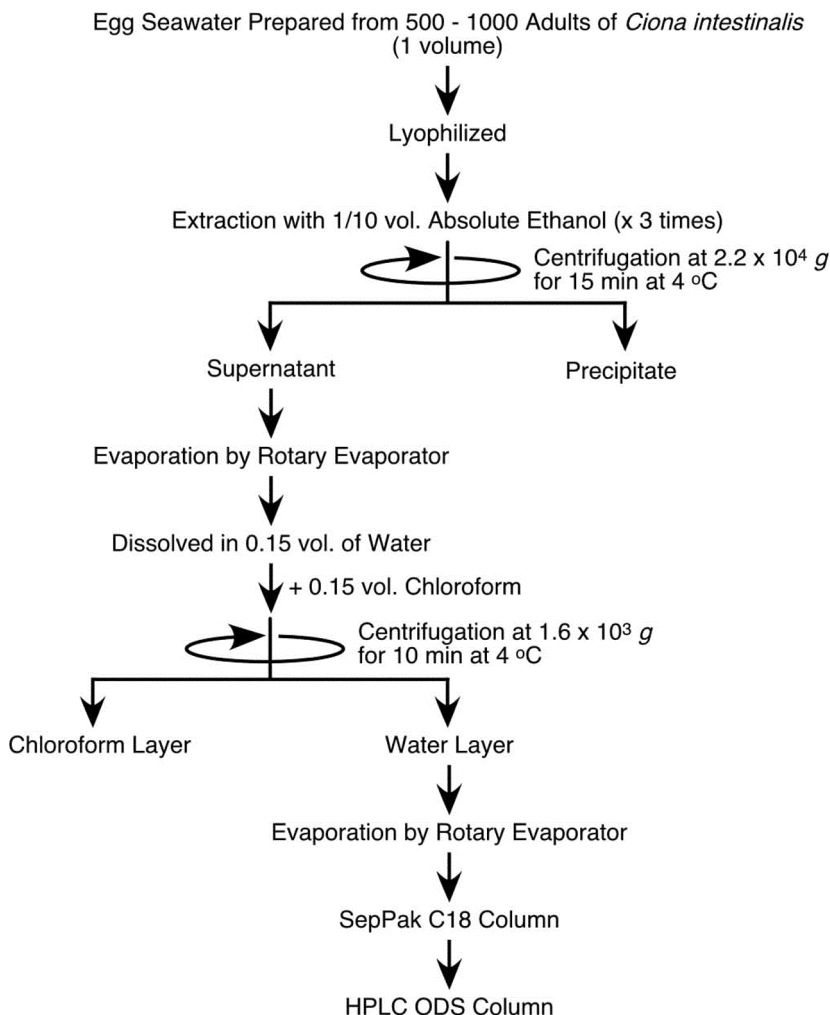


Fig. 4. Schematic drawing of purification procedures.

fresh tube. Perform ethanol extraction from the precipitate twice more and combine the obtained supernatant.

5. Evaporate the ethanol from the supernatant with a rotary evaporator.
6. Dissolve the residue in a volume of deionized water equal to the volume of ethanol used for the extraction.
7. Add an equal volume of chloroform and stir.
8. Centrifuge at $1.6 \times 10^3 g$ for 10 min. Transfer the upper water layer to a fresh tube.
9. Evaporate the layer with the rotary evaporator and dissolve the residue in the volume of deionized water equal to one-half of the volume of water used for dissolving the ethanol extract. This sample is the crude SAAF solution.

10. Column chromatography using reversed-phase resin, Sep-Pak C18.
 - a. Add 100 mL of methanol to 40 mL of Sep-Pak C18 resin (Waters, Milford, MA) and gently mix. Keep it for 5 min until the resin settles.
 - b. Discard the supernatants by decanting. Add 60 mL of methanol to the resin and gently mix it until the resin is uniformly suspended.
 - c. Place the empty column on the stand. Fill the column with methanol.
 - d. Immediately add a uniform suspension of Sep-Pak C18 resin to the column with a pipet or decantation. Wash the column with 200 mL of methanol. Then, equilibrate the column with 200 mL of deionized water.
 - e. Apply the crude SAAF solution from **step 9** to the Sep-Pak C18 column by decanting or pipetting. Discard the flowthrough.
 - f. Wash the column by adding 150 mL of deionized water. Discard the flowthrough.
 - g. Wash the column by adding 150 mL of 20% methanol. Discard the flowthrough.
 - h. To elute the adsorbed materials, add 150 mL of 60% methanol.
 - i. Dry the collected elute with a freeze-dryer or a rotary evaporator. Dissolve the residue in 1 mL of deionized water.
 - j. Check the sperm-activating and sperm-attracting activity.
11. Column chromatography using an ODS HPLC column.
 - a. Wash the C18 HPLC column with a 10-bed volume of 80% acetonitrile, and then equilibrate the column with a 10-bed volume of 20% acetonitrile.
 - b. Load the sample from **step 10** onto the HPLC column.
 - c. Wash the column with a 1-bed volume of 20% acetonitrile; then elute with a 5-bed volume of a 20–30% acetonitrile linear gradient. Collect fractions, dry the fraction with the centrifugal vaporizer, and dissolve the residue with 1 mL of deionized water.
 - d. Assay the sperm-activating and sperm-attracting activity of each fraction. Usually the activity is eluted at around 25–28% acetonitrile fractions. The obtained active fraction is a purified SAAF.

3.3. Observation of Sperm Chemotaxis

1. Wash glass slides thoroughly to remove oil drops on the surface. Dry the glass slides.
2. To prevent sperm sticking on the glass surface, coat glass slides with 2% BSA using a pipet (*see Note 2*).
3. Make the micropipets from 1-mm-outer diameter glass capillaries using a micropipet puller. From the shoulder to the tip, the length should be about 1 cm. If too long or too short, adjust the micropipet puller. Cut the tip of micropipets to 50–100 μm .
4. Mix each sample to examine the chemotactic activity with the same volume of 2% agar and keep the mixture at 50–60°C to prevent coagulation.
5. Put the micropipet into the mixture. The mixture should rise into the tip of the micropipet by capillary action. Leave the packed micropipet until the agar coagulates.

6. Set the micropipet on a micromanipulator.
7. Dilute the stocked semen to 1/10,000–1/5000 with ASW containing 1 mM theophylline and incubate for 1 min at room temperature for preactivation of sperm motility.
8. Set the glass slide on a phase-contrast microscope and put the sperm suspension on the BSA-coated glass slide.
9. Insert the micropipet in the sperm suspension. Set the tip of the micropipet in the center of the field of view.
10. Upload images of sperm around the micropipet tip onto a personal computer every 20 ms using a high-speed charge-coupled device (CCD) camera (HAS-200, Ditect; or similar instrumentation) and a video card (HAS-PCI, Ditect; or similar instrumentation) (*see Note 3*). To observe flagellar formation, set the electrical shutter speed to 1/500 s or faster.

3.4. Analysis of Sperm Chemotaxis

Analyze the data for position of each sperm obtained in **step 10** of **Subheading 3.3.** as follows:

1. Digitize the position of each sperm using an image-analyzing application (Dip-motion 2D, Ditect; or similar instrumentation). Locate the micropipet tip to the origin (0).
2. Calculate the distance between the micropipet tip and sperm head (D) for every point of the sperm as follows (*see Fig. 5*):

$$D = (X_t^2 + Y_t^2)^{1/2}$$

3. Plot the value for D against time. Calculate the LECI as a negative value of the coefficient ($-a$) in the linear equation $y = ax + b$ of the time-vs- D plots.
4. Calculate the differential quotient of D with respect to time (dD/dt) as $(D_{P2} - D_P)/Dt$ (*see Fig. 4* and **Note 4**).
5. Calculate the change of sperm direction (θ) as follows (*see Fig. 5*):

$$\theta = \pi - \arccos \frac{(x_{t+\Delta t} - x_t)^2 + (y_{t+\Delta t} - y_t)^2 + (x_t - x_{t-\Delta t})^2 + (y_t - y_{t-\Delta t})^2 - (x_{t+\Delta t} - x_{t-\Delta t})^2 - (y_{t+\Delta t} - y_{t-\Delta t})^2}{2\sqrt{(x_{t+\Delta t} - x_t)^2 + (y_{t+\Delta t} - y_t)^2} \sqrt{(x_t - x_{t-\Delta t})^2 + (y_t - y_{t-\Delta t})^2}} \text{ (rad)}$$

(*see Note 4*).

6. Examine each parameter. The chemotactic turn is represented as $\theta > \pi/3$ (60°).

4. Notes

1. A dark-field microscope is more appropriate to observe sperm flagella than a phase-contrast microscope. A phase-contrast microscope with a negative-phase lens (NH series lens in Olympus, or BM series lens in Nikon) is the most appropriate system for observing the sperm flagella, but it is very difficult to get these lenses now, because major microscope companies have stopped making this type of lens.

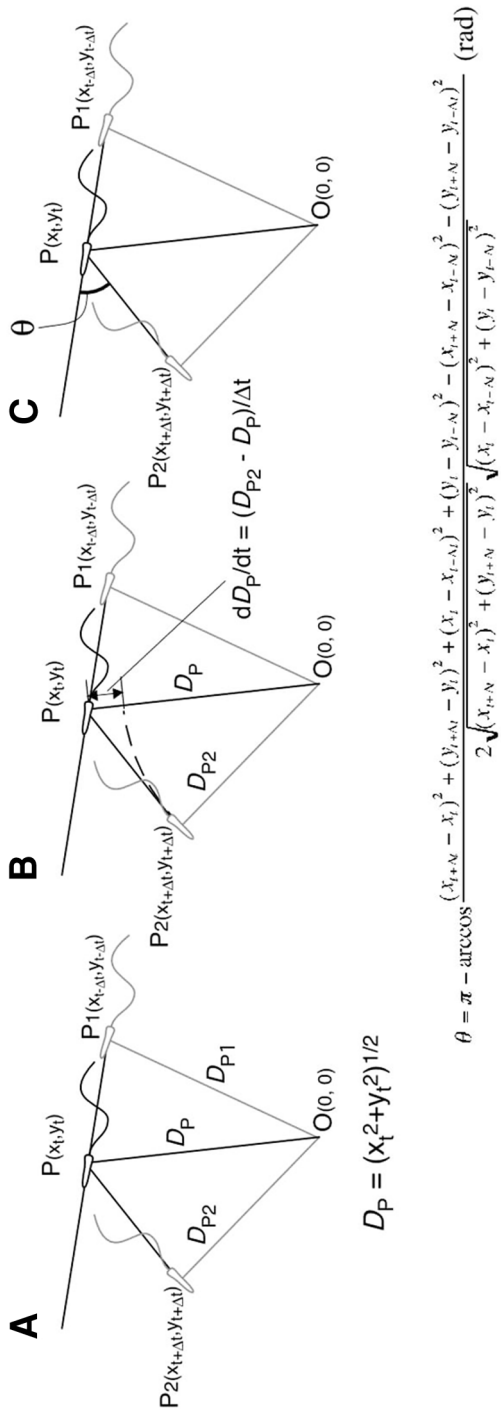


Fig. 5. Definition of parameters of the sperm chemotaxis: (A) D , (B) dD/dt , (C) θ . Formulas show the calculation method for each parameter of the sperm on the point $P(x_t, y_t)$. $P_1(x_{t-\Delta t}, y_{t-\Delta t})$ and $P_2(x_{t+\Delta t}, y_{t+\Delta t})$ represent the points of the sperm before and after Δt time, respectively.

2. To remove particles from the BSA, it is better to filtrate with a 0.45- μ m syringe filter.
3. The method of capturing the sequential images varies and depends on the High-speed video system. Please follow the instruction manual of your own system.
4. To smooth the sperm trajectories, it is better to configure Δt as 40–60 ms (two or three frames).

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