

Analysis of sperm chemotaxis

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Abstract

In many species, sperm must locate the female gamete to achieve fertilization. Molecules diffusing from the egg envelope, or the female genital tract, guide the sperm toward the oocyte through a process called chemotaxis. Sperm chemotaxis has been studied for more than 100 years being a widespread phenomenon present from lower plants to mammals. This process has been mostly studied in external fertilizers where gametes undergo a significant dilution, as compared to internal fertilizers where the encounter is more defined by the topology of the female tract and only a small fraction of sperm appear to chemotactically respond. Here, we summarize the main methods to measure sperm swimming responses to a chemoattractant, both in populations and in individual sperm. We discuss a novel chemotactic index (CI) to score sperm chemotaxis in external fertilizers having circular trajectories. This CI is based on the sperm progressive displacement and its orientation angle to the chemoattractant source.

1 Introduction

A sperm needs to swim toward the egg to be able to fertilize it. This process requires for this cell to detect and interpret chemical signals given by molecules diffusing from the egg outer layer, which guide the sperm toward the female gamete; a process

called chemotaxis. Sperm chemotaxis is a widespread phenomenon found in different species, from lower plants to mammals. Because of its fundamental role in fertilization, sperm chemotaxis has been studied for more than 100 years (Eisenbach, 1999; Miller, 1985; Pfeffer, 1884).

Sperm chemotaxis has been examined since 1884, when it was first described in ferns (Pfeffer, 1884). Animal sperm chemotaxis was not reported until 1912, when Lillie was studying the agglutinating factor present in the oocytes of the sea urchin *Arbacia punctulata*; he could observe sperm accumulation with the naked eye (Lillie, 1912, 1913). Again in fern spermatozoa, when Brokaw examined the sperm chemotactic response, he described the first chemoattractant molecule: the bimalate ion (Brokaw, 1957a, 1957b). Sperm chemotaxis has been studied in a great variety of species and several methods have been developed to measure it. Although the diversity of species with sperm chemotactic responses is quite broad, the mechanisms underlying this process are likely to be shared, at least to a certain degree, among them. Sperm chemotaxis has been characterized in external fertilizers, particularly in marine invertebrates, as their chemotactic responses are more prevalent in the sperm population than in internal fertilizers, where only a small fraction of spermatozoa responds (Ralt et al., 1994).

To navigate toward the egg, a sperm must regulate its flagellar movement according to the chemoattractant concentration field it detects while swimming. Flagellar motor changes control the sperm swimming trajectory, coupling the mechanical to the chemoattractant stimuli through complex signaling pathways (Wachten, Jikeli, & Kaupp, 2017; Yoshida & Yoshida, 2011). Imagine two different spermatozoa of the same species sensing a chemoattractant gradient in different localizations near an egg; both of them would detect and interpret the chemoattractant gradient in a particular manner, and thus each one will orient their swimming response differently. Therefore, studying individual sperm swimming trajectories has been fundamental to discriminate between different types of sperm responses, and key to understand how sperm chemotaxis is regulated (Miller, 1985).

Intracellular calcium ($[Ca^{2+}]_i$) is known to finely regulate sperm flagellar motor responses, altering how the axoneme generates mechanical forces (Brokaw, 1979; Guerrero et al., 2011; Kaupp, Kashikar, & Weyand, 2008; Mizuno et al., 2012). The detailed study and characterization of sperm trajectories has allowed to analyze and correlate several swimming parameters with changes in the $[Ca^{2+}]_i$. Although not fully understood, one of the best examples is the correlation between $[Ca^{2+}]_i$ oscillations and the turn-and-run chemotactic response in sperm from external fertilizers, which controls the chemotactic response in these species (Böhmer et al., 2005; Guerrero et al., 2010; Shiba, Baba, Inoue, & Yoshida, 2008; Yoshida, Murata, Inaba, & Morisawa, 2002).

Several experimental assays have been developed to study sperm chemotaxis, some of them focus on the analysis of population responses and other are tailored on the quantitative assessment of motility parameters as sperm swims in response to a given stimulus.

Table 1 Methods to analyze sperm chemotaxis.

Method	Animal/species	References
Trajectory curvature	<i>Ascidians</i> , <i>Siphonophores</i> , <i>Sea urchin</i>	Böhmer et al. (2005), Cosson, Carré, and Cosson (1984), Guerrero et al. (2010), Miller (1982), Shiba et al. (2005), Wood et al. (2007), Yoshida et al. (2002)
Asymmetry in the trajectory	<i>Sea urchin</i> , <i>Ascidians</i>	Böhmer et al. (2005), Cosson et al. (1984), Guerrero et al. (2010), Miller (1982), Shiba et al. (2005), Wood et al. (2007), Yoshida et al. (2002)
Linear equation chemotaxis index (LECI)	<i>Ascidians</i>	Yoshida (2004), Yoshida et al. (2002), Yoshida, Hiradate, Sensui, Cosson, and Morisawa (2013)
Accumulation assay	<i>Human</i> , <i>Rabbit</i> , <i>Mouse</i>	Eisenbach (1999), Fukuda, Yomogida, Okabe, and Touhara (2004), Gatica et al. (2013), Guidobaldi et al. (2017)
Accumulation assay (directionality based)	<i>Rabbit</i>	Burnett, Tholl, and Chandler (2011), Fabro et al. (2002)
Sperm dispersion (diffusion coefficient)	<i>Squid</i> , <i>Sea urchin</i>	Hirohashi et al. (2013), Inamdar et al. (2007)
Directionality angles	<i>Sea urchin</i> , <i>Abalone</i>	Brokaw (1979), Miller (1982), Shiba et al. (2008)
Mean vector length (orientation angle based)	<i>Human</i> , <i>Sea urchin</i> , <i>Abalone</i> , <i>Ascidian</i>	Gakamsky, Schechtman, Caplan, and Eisenbach (2008), Hussain, Sadilek, Salad, Zimmer, and Riffell (2017), Riffell, Krug, and Zimmer (2002), Riffell and Zimmer (2007), Spehr et al. (2003), Zimmer and Riffell (2011)
3D helix	<i>Sea urchin</i>	Crenshaw (1993a, 1993b), Crenshaw and Edelstein-Keshet (1993), Friedrich and Jülicher (2009), Friedrich and Jülicher (2007), Jikeli et al. (2015)

Table 1 summarizes the principal methods that have been used to date to measure sperm chemotaxis, in sperm from internal and external fertilizers. The majority of methods to estimate chemotaxis in internal fertilizers are scarce, and most of them quantify sperm accumulation. A classical experimental setup to score sperm drifting toward (or not) a chemical stimulus uses a two well Zigmond chamber, where spermatozoa are loaded in one well and the chemoattractant in the other, both wells are connected by a pathway where sperm cells can swim either toward the chemoattractant source, or away of it (Eisenbach, 1999). Most accumulation assays cannot distinguish between sperm chemotaxis, accumulation, swarming, chemokinesis, trapping and/or cell death. Nevertheless, Zigmond chambers have been also used to study chemo-orientation through the directionality-based accumulation assay,

which considers the single-cell trajectories while swimming to the chemoattractant well. With this approach, the average displacement of a population along the gradient direction is estimated from single-cell trajectories, by measuring the absolute sperm displacement alongside the horizontal (ΔX), vs the vertical (ΔY) axis of the Zigmond chamber, with the chemoattractants gradient aligned with the X axis of the chamber (Burnett et al., 2011; Fabro et al., 2002). Positive chemo-orientation responses are indicated by providing a coefficient $\Delta X/|\Delta Y| > 0$.

Sperm chemo-orientation, or biased motion scoring techniques, have been extensively employed to study the mammalian sperm in chemotactic assays, where due to the intrinsic natural variability of the samples, only a minor fraction of cells respond to the chemical stimulus (Gakamsky et al., 2008; Ralt et al., 1994). In such cases, where the chemo-orientation responses are subtle, the statistical measurements of odd ratios of swimming direction can be used to study the direction persistence of sperm swimming, hence providing a quantitative measurement of chemotactic performance (Armon, Caplan, Eisenbach, & Friedrich, 2012).

Another way to quantify chemotaxis in a population of spermatozoa is to measure sperm dispersion or agglutination, i.e., by studying the kinetics of cell density in response to the presence, or absence of a chemotactic stimulus. Directional based motion can be inferred from the local dynamics of cell density (Hirohashi et al., 2013; Inamdar et al., 2007).

Single individual cell analysis gives much more information about the sperm swimming behavior than the population assays. Because of this it became essential to track single-cell trajectories to characterize chemotaxis. Single-cell tracking and trajectory analysis was developed as the technology allowing the recording of individual sperm swimming behavior became available (Brokaw, 1957a, 1957b; Miller, 1985). Nowadays, technology allows analysis of each sperm cell by computer algorithms that segment the images composing a video, characterize automatically each sperm trajectory and calculate several swimming parameters (Table 1). In 1993, Crenshaw developed a mathematical framework to analyze chemotactic responses in 3D. He proposed the use of the central axis of the helix to calculate the orientation angle to its reference vector, which is perpendicular to the chemoattractant source (Crenshaw, 1993a, 1993b; Crenshaw & Edelstein-Keshet, 1993; Jikeli et al., 2015). Today it has become possible to determine individual swimming sperm trajectories and even flagellar movements in three dimensions (Corkidi et al., 2017; Jikeli et al., 2015; Pimentel & Corkidi, 2009; Su, Xue, & Ozcan, 2012).

Measuring swimming trajectory curvature and/or its asymmetry has been widely used as an indirect way to evaluate sperm swimming behavior in some species, as these swimming parameters indirectly reflect sperm changes in direction. Curvature and asymmetry are good indicators of abrupt changes in the swimming trajectory, but do not correctly follow small gradual changes that could be important in chemotactic responses. Most importantly, curvature and asymmetry are indirect measures of the changes in sperm swimming, but they do not reflect if the sperm direction is toward or away from the egg. Nevertheless, these physical parameters have been used to

evaluate the sperm swimming behavior (Böhmer et al., 2005; Cosson et al., 1984; Fukuda et al., 2004; Guerrero et al., 2010; Miller, 1982; Shiba et al., 2005; Wood et al., 2007; Yoshida et al., 2002).

There are other swimming parameters that reflect more precisely the sperm chemotactic behavior, as the directionality angles relative to the source of the chemoattractant, which measure the sperm orientation. A mean vector length was implemented by Riffell et al., 2002, which incorporates information about the orientation angles and calculates a unit vector that reflects the chemotactic behavior, having a value of 1 when there is chemotaxis, and a value of 0 when it does not occur (Table 1).

Another method to quantify sperm chemotaxis was proposed by Yoshida et al. (2002), to study the sperm motility response to egg jelly extracts from Ascidian species. Hence, a linear equation chemotactic index (LECI) was proposed, which is computed from the changes of the sperm head position in relation to the source of a chemotactic stimulus. LECI is obtained as the negative value of the coefficient ($-a$) in the linear regression model ($y = ax + b$) fitted to the distance of the sperm head to the source of the gradient across time (Figs. 2C, D and 3A, D; Movies 2 and 3 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>). Thus, a sperm cell approaching the source of the chemoattractant gradient (i.e., negative slope in the average speed) will have a positive LECI value. If the sperm is swimming away from the source (i.e., positive slope in the average speed), it will display a negative LECI value; and if the sperm is swimming in concentric circles in the same position (i.e., not responding), LECI will have a value of 0. A concern with the use of LECI as a way to quantify sperm chemotaxis is that it does not consider the sperm swimming behavior previous to chemoattractant stimulation.

Robust chemotactic responses, where the spermatozoon is swimming directly toward the egg, are easy to measure with the different methods described till now. However, when marginal chemotactic responses are involved, it becomes difficult to quantify with the methods described here so far. A more precise way to evaluate chemotaxis is needed.

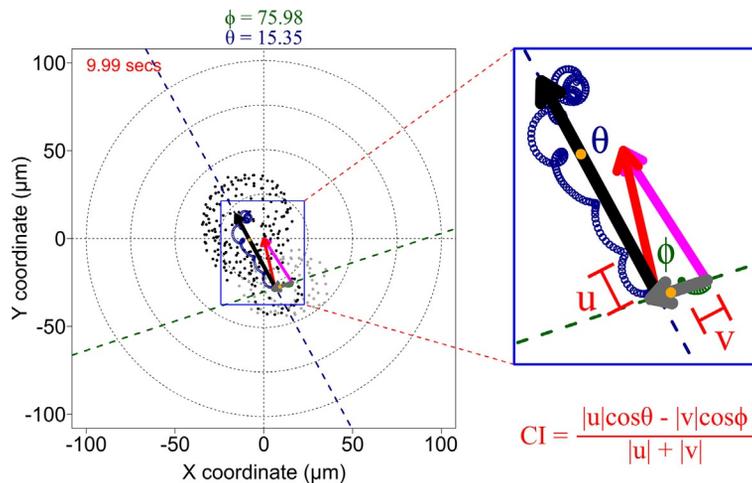
2 Measuring sperm chemotaxis

To better study and characterize the chemotactic behavior in single sperm cells, the use of photoactivating chemoattractants has provided important advantages (Böhmer et al., 2005; Guerrero et al., 2010; Tatsu, Nishigaki, Darszon, & Yumoto, 2002; Wood et al., 2007). These modified chemoattractants can be activated with a flash of a particular wavelength, allowing characterization of chemotactic responses to the diffusing chemoattractant in the absence of any external hydrodynamic perturbation, introduced by pipetting or perfusing the chemoattractant to the imaging chamber where the spermatozoa are swimming. Activating the chemoattractant without inducing any external force is crucial to analyze and quantify sperm chemotaxis in a model system.

Sperm trajectories of external fertilizers, like the sea urchin, are characterized by the turn-and-run typical response during the chemotactic swimming. These sperm display a thigmotactic circular component of the sperm trajectories when swimming close to the boundaries (Miller, 1985). This characteristic is wonderful to study chemotactic swimming responses but may be more complex to quantify them, in comparison to sperm species whose swimming is progressive during the chemotaxis process. As reviewed earlier, the different methods to quantify sperm chemotaxis in external fertilizers do not consider the unstimulated drift movement, the drift speed, the angle between the drift direction and gradient direction at the same time. Taking into account these potential problems and the fundamental concepts in measuring sperm chemotaxis developed by Crenshaw, Brokaw, Miller, Riffell and Yoshida (Brokaw, 1979; Crenshaw, 1993a, 1993b; Crenshaw & Edelstein-Keshet, 1993; Miller, 1982; Riffell et al., 2002; Yoshida et al., 2002), we have proposed a chemotactic index (CI), which incorporates information of all of these swimming parameters (Ramírez-Gómez et al., 2018) (Fig. 1 and Movie 1 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>).

This chemotactic index eliminates the circular component of a typical sea urchin sperm trajectory, smoothing the original trajectory using a moving average filter which represents, in number of frames, two complete unstimulated circles in the sperm trajectory (Fig. 1 and Movie 1 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>). Thereafter, a linear model is fitted to the smoothed trajectory and the corresponding line is forced to go through the mean point of the smoothed trajectory (orange point in Fig. 1 and Movie 1 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>) to guarantee it follows the direction of the trajectory. The chemotactic index is defined based on the progressive sperm displacement as
$$CI = \frac{|u|\cos\theta - |v|\cos\phi}{|u| + |v|}$$
, being ϕ and θ the orientation angles, and $|v|$ and $|u|$ the progressive speed (i.e., drift speed), before and after the chemoattractant activation, respectively. The orientation angles represent the angles between the progressive sperm displacement and the reference vector, which points to the center of the field where the highest chemoattractant concentration could be found. The orientation angles can take values from 0° to 180° , depending on if the sperm is swimming toward or away from the chemoattractant concentration field, respectively (Fig. 1). The magnitude of the progressive sperm displacement is calculated changing the coordinate system from the smoothed trajectory to the linear regression model (i.e., from two dimensions to one dimension), collapsing the smoothed trajectory in the linear model and then tracing a vector along the length of transposed coordinates, before (gray vector) and after (black vector) the chemoattractant activation; the progressive speed is defined as the progressive sperm displacement divided by the time.

This chemotactic index considers the progressive sperm displacement before the chemoattractant activation (i.e., unstimulated drift movement) and then subtracts it

**FIG. 1**

Chemotactic index (CI). Definition of a chemotactic index to quantify single-cell chemotactic responses. Dots represent sperm trajectory before (gray) and after (black) chemoattractant activation. Green and blue spirals indicate the smoothed trajectory before and after chemoattractant activation, respectively. Progressive sperm displacement before (gray vector) and after (black vector) chemoattractant activation and progressive speed before ($|v|$) and after ($|u|$) chemoattractant activation are shown in the inset; and ϕ and θ are the angles between the progressive sperm displacement and their corresponding reference vectors, pointing to the center of the imaging field—the highest chemoattractant concentration (magenta and red vectors, respectively). The chemotactic index (CI) is defined as in the inset. The orange point represents the mean point of the smoothed trajectory, before and after chemoattractant activation. See Movie 1 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>.

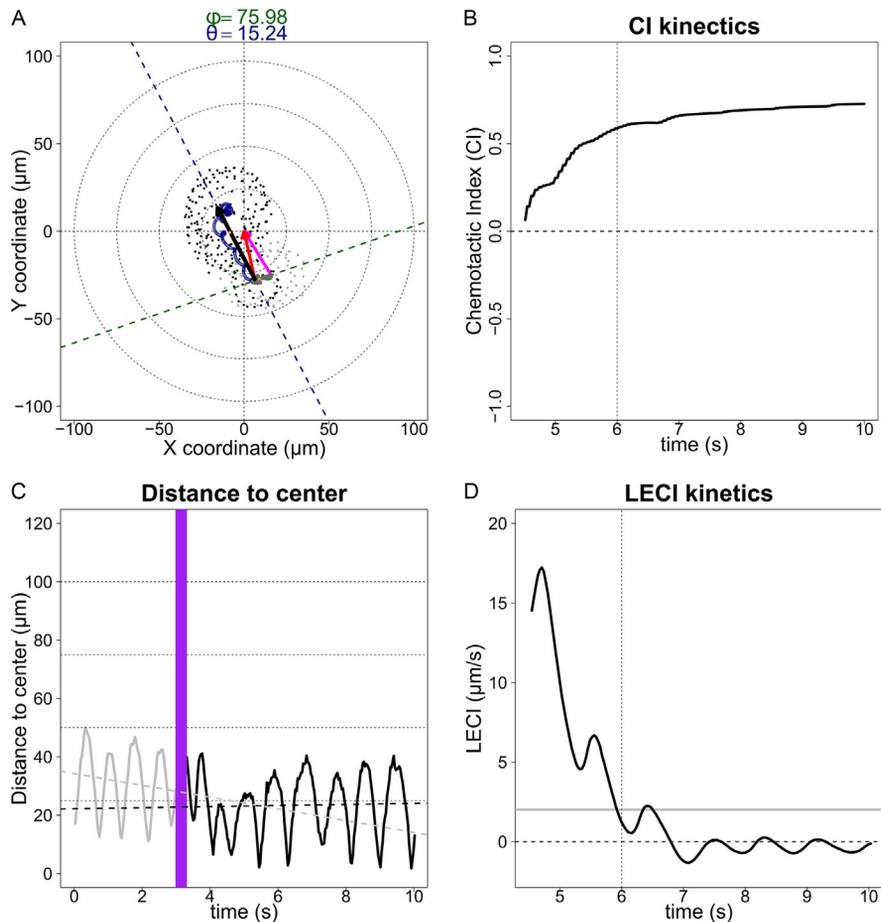
from the sperm chemotactic response, eliminating the possibility of scoring a chemotactic index biased by the unstimulated drift movement, previous to the chemoattractant activation. For the cases where the unstimulated sperm is swimming in concentric circles with a small drift movement, the progressive sperm displacement before the chemoattractant activation would have almost no contribution to the CI. On the other hand, if the unstimulated drift movement is big, it would considerably impact the value of the CI depending on the size of the chemotactic response, and on the orientation angles before and after the chemoattractant activation. This CI score values go from -1 to 1 , indicating negative and positive chemotaxis, respectively; being 0 no chemotaxis at all. For the simple case, imagine that a positive chemotactic sperm is swimming toward a chemoattractant concentration field with an orientation angle close to 0° , so the cosine value will be about 1 (i.e., $\cos(0) = 1$). Now imagine a

negative chemotactic sperm swimming away from a chemoattractant concentration field with an orientation angle close to 180° , so the cosine value will be about -1 (i.e., $\cos(\pi) = -1$). For the case of a non-chemotactic sperm swimming with no particular preference in a chemoattractant concentration field, moving perpendicular to the chemoattractant gradient direction, the cosine value will be about 0 (i.e., $\cos\left(\frac{\pi}{2}\right) = 0$). Besides the orientation angles, this CI is composed as a weighted average of the individual contributions of the progressive sperm displacement, before and after the chemotactic response.

As indicated earlier in this chapter, the linear equation chemotactic index (LECI) is a precise method to quantify sperm chemotaxis (Yoshida et al., 2002). Nevertheless, this method is not robust enough to evaluate chemotaxis when a sperm is swimming in a subtle way toward the source of the chemoattractant concentration field, as the magnitude of LECI is itself a measure of the approaching velocity of the sperm head from/to the source of the chemoattractant gradient (Fig. 2A and Movie 2 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>). As it can be seen in Fig. 2D, with a marginal chemotactic trajectory (Fig. 2A), LECI fails to reflect the chemotactic behavior of the sperm all along the chemotactic response. For example, LECI will reflect a chemotactic behavior only when the sperm is moving with progressive speed (Fig. 2D, seconds 4.5 to 6), but in this case if chemotaxis is evaluated in this sperm after the second 6 it would be biased, indicating a non-chemotactic response when the LECI value is almost 0. Nevertheless, analyzing the same sperm with the proposed chemotactic index (CI) would score a positive chemotactic value at all times during a given chemotactic response (Fig. 2B). Fig. 3D and Movie 3 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002> show, as mentioned before, that robust chemotactic responses yield positive LECI values reflecting a chemotactic behavior at all times.

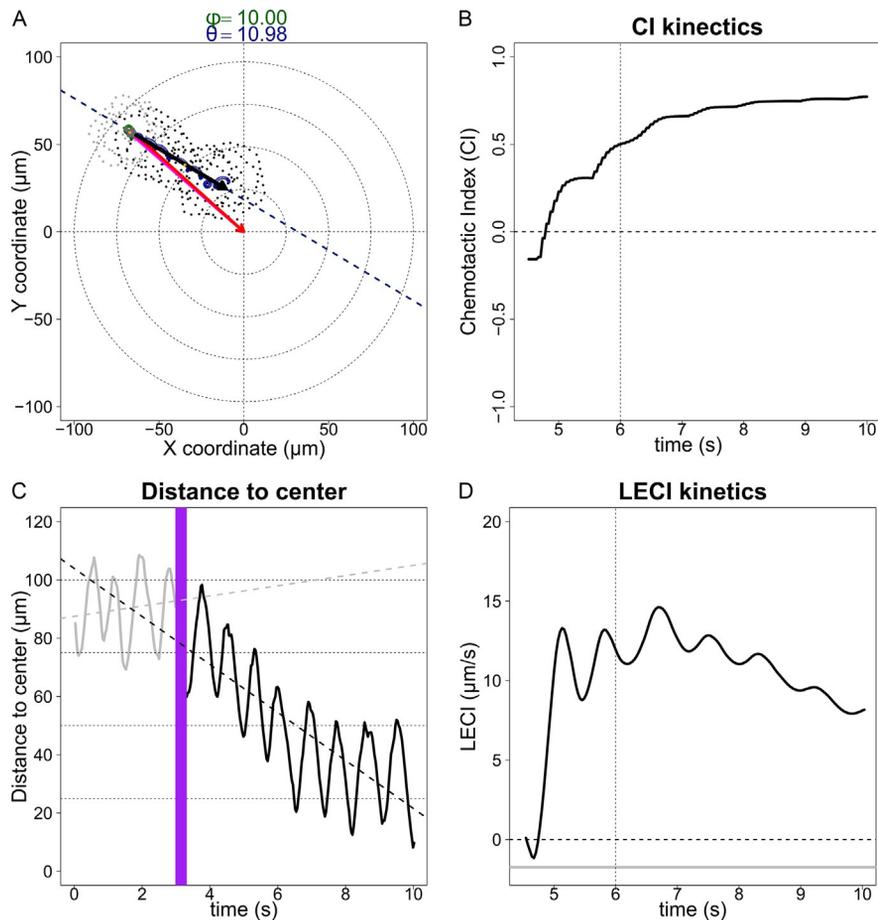
All these algorithms can be computed and all swimming parameters automatically calculated for each video. This allows evaluation of the temporal evolution of these chemotactic indexes for each individual sperm trajectory (Movies 1–3 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>). The analysis of swimming parameters in time, for tens or hundreds of sperm trajectories, generates large amounts of data that must be analyzed using statistical tools. We have developed this chemotactic index algorithm and statistical tools to analyze large amounts of data using the R programming language (R Core Team, 2016). We believe that this novel chemotactic index (CI) is more precise in quantifying sperm chemotaxis in external fertilizers, because it corrects the unstimulated drift movement, and considers the drift speed and the orientation angles between drift direction and chemoattractant gradient direction. As a matter of fact, we have used this algorithm and the statistical tools in a study, to discriminate between chemotactic responses of about 1000 sea urchin sperm, swimming in different chemoattractant gradient conditions (Ramírez-Gómez et al., 2018).

An interesting perspective that arises from this novel CI is to explore its application to examine chemotaxis in 3D since, as mentioned earlier, there are now several strategies to characterize swimming trajectories in free swimming spermatozoa.

**FIG. 2**

Comparison of chemotactic indexes in a marginal chemotactic response. (A) Marginal chemotactic response. Dots represent sperm trajectory before (gray) and after (black) chemoattractant activation. Green and blue spirals indicate the smoothed trajectory before and after chemoattractant activation, respectively. Progressive sperm displacement before (gray vector) and after (black vector) chemoattractant activation; and ϕ and θ are the angles between the progressive sperm displacement and their corresponding reference vectors. The orange point represents the mean point of the smoothed trajectory, before and after chemoattractant activation. (B) Temporal evolution of the chemotactic index (CI) of the sperm trajectory shown in (A). Note that the temporal evolution of the CI for this marginal chemotactic response always scores a positive chemotactic index. (C) Distance to the center and linear regression. Distance of the sperm head to the center of the field is plotted across time, before (gray) and after (black) chemoattractant activation (purple band), and the negative of the slope of the linear regression model (LECI) is calculated every frame of the video. (D) Temporal evolution of the linear equation chemotactic index (LECI) of the sperm trajectory shown in (A). Note that the temporal evolution of the LECI for this marginal response scores a non-chemotactic index (~ 0) from second 6 to second 10. This analysis was implemented from 4.5 to 10s. See Movie 2 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>.

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**FIG. 3**

Comparison of chemotactic indexes in a robust chemotactic response. (A) Robust chemotactic response. Dots represent sperm trajectory before (gray) and after (black) chemoattractant activation. Green and blue spirals indicate the smoothed trajectory before and after chemoattractant activation, respectively. Progressive sperm displacement before (gray vector) and after (black vector) chemoattractant activation; and ϕ and θ are the angles between the progressive sperm displacement and their corresponding reference vectors. The orange point represents the mean point of the smoothed trajectory, before and after chemoattractant activation. (B) Temporal evolution of the chemotactic index (CI) of the sperm trajectory shown in (A). (C) Distance to the center and linear regression. Distance of the sperm head to the center of the field is plotted across time, before (gray) and after (black) chemoattractant activation (purple band), and the negative of the slope of the linear regression model (LECI) is calculated every frame of the video. (D) Temporal evolution of the linear equation chemotactic index (LECI) of the sperm trajectory shown in (A). Note that both indexes (CI and LECI) score a positive chemotactic index with a robust chemotactic response. This analysis was implemented from 4.5 to 10s. See Movie 3 in the online version at <https://doi.org/10.1016/bs.mcb.2018.12.002>.

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