Cross-kymography analysis to simultaneously quantify the function and morphology of the archaellum

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In many microorganisms helical structures are important for motility, e.g., bacterial flagella and kink propagation in Spiroplasma eriocheiris. Motile archaea also form a helical-shaped filament called the ‘archaellum’ that is functionally equivalent to the bacterial flagellum, but structurally resembles type IV pili. The archaellum motor consists of 6–8 proteins called fla accessory genes, and the filament assembly is driven by ATP hydrolysis at catalytic sites in FlaI. Remarkably, previous research using a dark-field microscopy showed that right-handed filaments propelled archaeal cells forwards or backwards by clockwise or counterclockwise rotation, respectively. However, the shape and rotational rate of the archaellum during swimming remained unclear, due to the low signal and lack of temporal resolution. Additionally, the structure and the motor properties of the archaellum and bacterial flagellum have not been precisely determined during swimming because they move freely in three-dimensional space. Recently, we developed an advanced method called “cross-kymography analysis”, which enables us to be a long-term observation and simultaneously quantify the function and morphology of helical structures using a total internal reflection fluorescence microscope. In this review, we introduce the basic idea of this analysis, and summarize the latest information in structural and functional characterization of the archaellum motor.

Key words: archaea, archaellum, bacterial flagellum, TIRFM, cross-kymography analysis

The motility of organisms is driven by nano-sized molecular machines that convert chemical energy into mechanical work. Eukaryotes (whether myosin, kinesin or dynein) use the free energy of ATP hydrolysis and are responsible for intracellular work such as muscle contraction and cell division [1]. A representative example of bacterial motility is the flagellar rotation of Escherichia coli and Salmonella enterica serovar Typhimurium, which consists of about 30 different kinds of proteins and is attached to the helical filament via the hook structure [2]. The flagellar filament exits outside the bacterial cell body, allowing the cell to swim by rotating the filaments like a screw using the free energy of the ion gradient.

The ‘conventional’ motile systems of these molecular motors have been studied extensively over the last several decades using various approaches such as crystal structure, gene manipulation, and functional analysis with single-molecular techniques. Recently, forms of motility in bacteria without flagella have also been clarified, such as pili that pull...
[3–5], Mycoplasma legs that walk [6–8], and internal structures that contort [9,10]. In this review, we highlight the motility of the third domain, ‘Archaea’; i.e., the swimming motility driven by archaellar rotation [11]. Additionally, we describe a novel assay called ‘cross-kymography analysis’, which we use to quantify the morphology and function of helical structure in real time [11,12].

Archaea

Archaea were initially regarded as extreme-environment microorganisms. This was because halophilic archaea such as Halobacterium salinarum and Haloferax volcanii in Euryarchaeota require a few M of salt for cultivation, and the highly thermophilic archaeeon Sulfolobus acidocaldarius in Crenarchaeota needs more than 60°C to grow [13]. However, archaea have recently been found in normal environments such as sea and soil [14,15]. The shape of archaea is very similar to that of bacteria, and archaea were regarded as identical to bacteria by the end of the 20th century. In 1977, Woese and Fox showed that life forms could be divided into more than prokaryotes and eukaryotes, based on the results of 16 S ribosomal RNA analysis [16]. In 1990, Woese et al. proposed that the living world consists of three domains: eukaryote, bacteria, and archaea [17].

Archaellum

The swimming archaea form a helical filament on the cell surface like swimming bacteria (Fig. 1a and b). In 1984, Alam and Oesterhelt used a dark-field microscope to demonstrate that right-handed filaments propel cells forwards or backwards by rotating filaments in clockwise and counterclockwise directions, respectively [18]. A motility machine of archaea had been thought to look similar to the bacterial flagellum, not only in function but also in structure. However, DNA sequence analyses have shown that archaea lack genes encoding the ring structures, the rod, and the hook,

Figure 1 Motility structure of archaea, archaellum
(a) Electron micrograph of Halobacterium salinarum cell (left) and magnified image of archaella (right). Scale bars, 2 μm (left) and 0.1 μm (right).
(b) Electron micrograph of Escherichia coli cell (left) and magnified image of flagella (right). Scale bars, 2 μm (left) and 0.2 μm (right).
(c) The organization of archaella operon. Homologous genes are represented in the same colors.
(d) The current model of archaellar motor. Pre-archaellin has a short signal sequence, and the polymerization proceed after removing this signal sequence by the signal peptidase FlaK/PibD in Euryarchaeota and PibD for Crenarchaeota. In Crenarchaeota, archaellar filament is composed of a single archaellin, FlaB, whereas Euryarchaeota possess the several archaellin genes, flaA or flaB. FlaB3 might play role of hook in Euryarchaeota. Motor is composed of a conserved the membrane protein (FlaJ), the hexametric ATPase (FlaI) and the regulator of ATPase activity (FlaH). The C-terminal domain of FlaI contains the Walker A and B motif for ATP-binding and hydrolysis, and its ATPase activity is essential for not only archaellum assembly and rotation. Nine to ten FlaH molecules assemble in a ring complex and regulates the ATPase activity of FlaI. In addition, Euryarchaeota form FlaC/D/E for motor complex, whereas Crenarchaeota possess FlaX instead. FlaC/D/E play a role for the control of rotational direction by interacting with Che proteins.
which are part of the bacterial flagellum. Interestingly, archaea do have operons which are a similar in sequence to archaenal and bacterial type IV pili, which serve many purposes such as twitching motility, adhesion, biofilm formation and DNA uptake [19,20]. Some of the motor components of the archaenal flagellum show homologies to components of the type IV pilus assembly apparatus [19,20]. Despite being functionally the same, the gene sequence of archaenal flagellum is completely different from that of bacterial flagellum. To prevent confusion and distinguish the bacterial and archaenal flagellum, Jarrell and Albers proposed that archaenal flagellum should be renamed “archaellum” [20]. This name was initially controversial [21,22], but has gradually been accepted; therefore, we use the archaellum in this review.

**Filament assembly**

The archaellum motor consists of 6 different kinds of proteins in Crenarchaeota and 8 proteins in Euryarchaeota called fla accessory genes; it is attached to the helical archaenal filament (Fig. 1b and c). Electron microscopic observation revealed that some Euryarchaeota, though not all, formed the curved hook, and that the helical filament is connected with a motor, as seen in bacterial flagella [23].

The archaellum, the filament protein, has a short signal sequence, and is assembled upon signal peptide removal by the signal peptidase FlaK/PibD in Euryarchaeota, and PibD in Crenarchaeota [19]. In Euryarchaeota the filament can consist of several archaellins as seen in many kinds of flagellated bacteria such as Caulobacter crescentus, Vibrio fischeri, and Helicobacter pylori, etc [24–27]. The archaellum of Methanococcus voltae consists of the major archaellins flaB1 and flaB2 and a minor archaellin called flaA; additionally, flaB3 plays the role of hook. The deletion mutant of flaB3 formed archaenal filaments, which did not build a hook, suggesting that the hook proteins are assembled after filament polymerization [28]. This property is one of characteristics that sets the archaellum apart from the bacterial flagellum [2]. Furthermore, a recent study showed that the swimming motility of Haloferax volcanii was improved by the deletion of the minor archaellin flgA2, suggesting that flgA2 regulates the motility and function of the major archaellin flgA1 [29]. Thus far, the reason why archaea possess several archaellins remains elusive, but it is theorized that archaea might form multiple archaellins to stabilize the archaellum structure and function in adapting to extreme environments [30].

**Motor complex**

All archaella operons consist of FlaF and FlaG, a transmembrane protein Flaj, the hexametric ATPase Flai, and the regulator of ATPase activity Flai [19,20]. FlaF is assumed to act as stator of the archaenal motor, as it was found to interact with the S-layer proteins which are the archaenal outmost cell envelope [31]. Flai, a homolog of PilC in type IV pili, is an integral membrane protein and thought to form the archaenal motor platform [32]. The AAA’ ATPase FlaL possesses a Walker A, Walker B motif and a P-loop region, and has a significant homology to PilB in type IV pilus and to GspE in the type II secretion system [33]. The hexametric ring structure of Flai had a diameter of 14 nm, and the difference of the height between the ATP-bound and ADP-bound states was 1 nm. The Flai mutant did not form filaments at the cell surface, indicating that Flai is essential for the filament assembly [34,35]. Furthermore, a deletion of the first 29 amino acids of Flai leads to a non-motile phenotype although the archaellins are formed on the cell body, indicating that the N-terminal region of Flai is responsible for archaellin motor rotation [29]. FlaiH possesses conserved Walker A motifs, and interacts with Flai. Remarkably, the RecA domain of FlaiH is similar to the clock protein KaiC, suggesting that FlaiH might regulate the timing of rotation and archaellum polymerization [36].

Additionally, Euryarchaeota express FlaC/D/E which possibly interact with the motor complex, whereas Crenarchaeota possesses Flax instead [19]. Flax/D/E plays a role in the control of rotational direction by interacting with Che proteins [37]. The purified Flax from Sulfolobus acidocaldarius forms a 30-nm diameter ring structure, and might be essential for stabilization of FlaiH, Flai, and FlaiJ [38]. Indeed, 9–10 FlaiH molecules assemble inside a Flax ring complex in vitro [36].

**Swimming motility**

Archaellar rotation was first reported in 1984, but the shape and rotational rate of the archaellum during swimming remained unclear due to the low signal and lack of temporal resolution by a dark-field microscopy [18]. To overcome these problems, we stained the archaellar filaments with a fluorescent dye, Cy3, by biotin-avidin interaction (Fig. 2a). In this study, we used biotin with a long linker between the biotin head that recognizes avidin and the reactive group that recognizes the amino group. We succeeded in visualizing archaellar filaments with a high spatiotemporal resolution up to 2 ms. Remarkably, we also observed the two previously reported swimming modes of archaellar filament(s): pushing and pulling of the cell body [18]. Using kymography analysis, the swimming speed of cells and rotational rate of archaellar filaments were quantified from the green slope and number of pink dots (Fig. 2b). In pushing and pulling archaella, the ‘swimming speed/rotation rate’ were 2.9±0.5 μm s⁻¹/24.4±2.7 Hz (n=90) and 1.7±0.7 μm s⁻¹/21.7±2.0 Hz (n=44), respectively (Fig. 2c and d). The ratios of swimming speed to archaellar rotation in pushing and pulling were thus calculated to be 0.126 and 0.081 μm s⁻¹/Hz (n=44), respectively, indicating that propulsion distance per rotation of archaellum was almost similar to that of bacterial flagellum [12,39].
with the evanescent field was visible as short lines aligned northwest-southeast relative to the major axis of the cell body, indicating right-handed helicity. In our assay, only cells with right-handed helix were detected, without exception. In addition to determining archaellar helicity, our assay enabled us to quantify structural parameters such as the archaellar pitch, pitch angle, and helix radius from the still image. By drawing the green line along the archaellar filaments in Fig. 3c, multiple peaks could be detected corresponding to the archaellar pitch (Fig. 3d). The pitch angle was directly determined from the still image as the shallow angle of the archaella (pink line in Fig. 3c). With these values, the helix radius could be estimated with the following equation:

$$r = \frac{1}{2\pi \times \lambda \times \tan \theta}$$

where $r$ is the helix radius, $\lambda$ is pitch, and $\theta$ is the pitch angle. From this analysis, the pitch, pitch angle, and helix radius were quantified to be $2.1 \pm 0.2 \mu m$, $34 \pm 5^\circ$, and $0.22 \pm 0.03 \mu m$, respectively (Fig. 3e–g). Furthermore, our assay could quantify the rotation rate precisely. By fixing on the pixels where the archaella were rotating (blue square in Fig. 3c), oscillation of the intensity could be detected due to the archaellar oscillation (Fig. 3h). Because a diameter of archaellar filaments is ~400 nm, which is a few times wider than the penetration depth of the evanescent field. Using a Fourier transform analysis, the rotational rate of archaellar filaments was estimated to be $22.5 \pm 4.5$ Hz (Fig. 3i).
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right-handed filament rotates in the CCW direction. Similarly, we could determine the direction of wave propagation when left-handed filaments rotated in either direction. We schematically summarized the relationship between rotational direction and helicity (Fig. 4c); consequently, the relationship can be uniformly determined from four patterns. Using this technique, we observed that the right-handed filamentous of *Hbt. salinarum* rotated in CW direction (Fig. 4d) and CCW direction (Fig. 4e); additionally, the left-handed filamentous of *S. typhimurium* also rotated in CCW direction (Fig. 4f) and in CW direction (Fig. 4g). Furthermore, we first demonstrated that the helicity of the archaellum is right-handed, even if the motor switches the direction of rotation, which was in stark contrast to the feature of bacterial flagellar filament.

Future prospects

We constructed the cross-kymography analysis under TIRFM, and expect that this technique will be a breakthrough not only for the study of archaenal motility, but also for bacterial motility research [11–12]. Flagellated bacteria show motility by swimming towards a better environment, changing rotational direction and transforming flagellar helicity [2]. Long-term observation systems have been gradually developed; however, these methods are specialized for quantification of functional parameters such as rotational rate of filaments, not flagellar structures [40–42]. Our method could reveal “change of rotational direction of flagella” and “trans-
helpful in clarifying the novel motility mechanisms of unconventional bacteria by simultaneously monitoring the function and morphology of helical structures. Finally, microscopic measurements using an optical microscope have gradually clarified the function of the archaeal motor. However, many big mysteries remain, e.g., why archaeal filaments maintain right-handedness, and how motor switch and torque is generated at a molecular level. We expect that biophysicists will join this new research field and tackle these problems with various approaches, including crystal struc-

**Figure 4** Simultaneous determination of the helicity and rotational direction of archaellum by cross-kymography analysis
(a) The schematics of relationships between the direction of rotation (clockwise, CW; counterclockwise, CCW) and helicity (right-handed, RH; left-handed, LH). We first defined the configuration as archaella aligned parallel to the north–south orientation and the cell body located at the north; additionally, the rotation direction is defined as the observer looking at the archaella from the protrusion direction outside the cell body. Note that a camera is set on the left-side camera port, and images are always mirror images because the totally reflecting prism inside the inverted microscope reflects, just once, the light coming through the objective. The portion of filaments that made contacts with the evanescent field was visible as short lines aligned northwest-southeast relative to the major axis of the cell body, indicating the right-handed helicity. On the contrary, the orientation of filaments is northeast-southwest, indicating the left-handed helicity. (b) The schematics to explain how to determine the helicity from the east-west kymograph. Cyan and pink arrow indicated the direction of wave propagation. Gray, orange, magenta and green ellipse represents the time course of wave propagation. Therefore, we could detect the transition of fluorescent signal at the rectangle as shown by orange or green arrows. (c) Left: definition of axes. Right: matrix of combinations to determine the directional rotation and helicity of helical structure from cross-kymography, which is the analysis of two kymographs from two different lines in the same image that perpendicularly align with each other in the region of interest. (d-g) Left: Fluorescent micrograph. Right: two kymographs taken from orthogonally oriented lines on left micrograph. In north-south kymograph, the right-handed archaellar wave propagates away (d) and towards (e) the cell body, which guaranteed the CW and CCW rotation, respectively. On the contrary, the left-handed flagellar wave propagates away (f) and towards (g) the cell body, which guaranteed the CCW and CW rotation, respectively. In the east-west kymograph, the helicity could be uniformly determine as mentioned above. Pseudo-colors of kymographs indicate the direction of spot propagation, which correspond to c. The figure was reused with permission from Kinosita et al., 2016 with modifications.

formation of flagellar shape by switching simultaneously in real time and also verify the model that the flagellar filament modulates the switching frequency of the motor [43].

There are many spiral-shaped bacteria in nature; e.g., *Spiroplasma eriocheiris*. It shows a swimming motility in highly viscous environments by propagation of kink pairs along the cell body from front to back [44]. This propagation is considered to be driven by rotation, but there are unclear points on how the rotation is generated and transforms the helical structure of the cell body [45]. Our method will be
ture, cryo-electron microscope, and the single-molecular approach.

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Conflicts of interest

The authors declare no competing financial interests.

Author contribution

Y. K and T. N wrote the paper.

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