1993

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Recommended Citation

Hoops H.J. (1993) Flagellar, cellular and organismal polarity in Volvox carteri. Journal of Cell Science 104: 105-117. doi:

Journal of Cell Science 104, 105-117 (1993)
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Flagellar, cellular and organismal polarity in *Volvox carteri*

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SUMMARY

It has previously been shown that the flagellar apparatus of the mature *Volvox carteri* somatic cell lacks the 180˚ rotational symmetry typical of most unicellular green algae. This asymmetry has been postulated to be the result of rotation of each half of the flagellar apparatus. Here it is shown that *V. carteri* axonemes contain polarity markers that are similar to those found in *Chlamydomonas*, except that in *V. carteri* the number one doublets do not face each other as they do in *Chlamydomonas* but are oriented in parallel and at approximately right angles to the line that connects the flagella. Thus, the rotational orientations of the axonemes are consistent with the postulate that the flagella of *V. carteri* have rotated in opposite directions, as was predicted earlier from the positions of the basal fibers and microtubular rootlets. Moreover, high-speed cinephotomicrographic analysis shows that the *V. carteri* flagellar effective strokes are also oriented in approximately the same direction, and in parallel planes. These results suggest that the direction of the effective stroke in both *Chlamydomonas* and *Volvox* is fixed, and that rotation of the axoneme is the cause of the differences in flagellar motility observed between *Chlamydomonas* and *Volvox*. These differences are probably essential for effective organismal motility. Cellular polarity of *V. carteri* can be related to that of *Chlamydomonas* after taking into account the developmental reorientation of flagellar apparatus components. This reorientation also results in the movement of the eyespot from a position nearer one of the flagellar bases to a position approximately equidistant between them. By analogy to *Chlamydomonas*, the *anti* side of the *V. carteri* somatic cell faces the spheroid anterior, the *syn* side faces the spheroid posterior. The *cis* side of the cell is to the cell's left (the right to an outside observer), although it cannot be described solely on the basis of eyespot position as it can in *Chlamydomonas*, while the *trans* side is to the cell's right. It follows that if the direction of the effective flagellar stroke is specified by structural features, then effective organismal motility in *V. carteri*, will be accomplished only if the cells are held in the proper orientation with respect to one another. The simplest arrangement that will yield both progression and rotation in ovoid or spherical colonies composed of biflagellate isokont cells is one in which the cells are arranged with rotational symmetry about the anterior-posterior axis of the spheroid. Analysis of the polarity of somatic cells from throughout the spheroid shows that it is constructed with just such symmetry. This symmetry probably originates with the very first divisions.

Key words: *Volvox, Chlamydomonas,* asymmetry, cell polarity, flagellar motility, axoneme

INTRODUCTION

Volvox, like other colonial and multicellular members of the Volvocaceae, is sometimes viewed as a multicellular *Chlamydomonas* because of overall similarities in cell organization. Such similarities are presumed to reflect the evolution of these forms from a unicellular volvocalean ancestor (Crow, 1918; Shaw, 1919; Fritsch, 1929). Additional support for this view has been derived from observed similarities in mitosis and cytokinesis (Johnson and Porter, 1968; Coss, 1974; Triemer and Brown, 1974; Deason and Darden, 1971; Birchem and Kochert, 1979; Hoops and Floyd, 1982), cell wall structure (Roberts, 1974; Adair et al., 1987; Goodenough and Heuser, 1988; Matsuda, 1988), and of ribosomal RNA sequences (Rausch et al., 1989; Buchheim and Chapman, 1991; Larson et al., 1992). However, the unicellular and colonial members of the order also differ in a number of important respects. One such difference concerns the pattern of flagellar activity. In *Chlamydomonas* and other biflagellate, unicellular members of the Volvocales, the two flagella usually beat in a breaststroke-like motion, wherein the effective strokes of the flagella are oriented in opposite directions within a single plane. This propels the *Chlamydomonas* cell forward along its own cellular axis. However, in colonies composed of radially arranged cells, such motion would result in little or no motility. Gerisch (1959) showed that the flagella of *Pleodorina* cells beat with their effective strokes angled towards the colonial posterior; such a beat pattern has now been verified by high-speed cinephotomicrography of a number of species (Hoops and Floyd, 1983; Taylor et al., 1985; Lewis et al., unpublished).

The flagellar apparatuses of the mature unicellular and colonial volvocalean algae are also organized differently. The two halves of the flagellar apparatus of the unicellular Volvocales, including *Chlamydomonas*, are arranged with 180˚ rotational symmetry (Floyd et al., 1980). This type of symmetry is of a very general occurrence; three of the four classes of advanced green algae in the classification scheme of Mattox and Stewart (1984) display this type of symmetry, as do the immature cells of colonial and multicellular Volvocales (Hoops and Floyd, 1983; Hoops, 1984; Taylor et al., 1985; Greuel and Floyd, 1985). During cleavage, the basal bodies of colonial and multicellular forms are without flagella, but flagella begin to grow from the basal bodies before hatching and while the flagellar apparatus still retains 180˚ rotational symmetry. Subsequently, however, the flagellar apparatus undergoes substantial reorganization before the embryo breaks free of the parent spheroid. In every case yet examined, except that of the central cells of *Gonium pectorale*, this reorganization results in the loss of 180˚ rotational symmetry (Hoops and Floyd, 1983; Hoops, 1984; Taylor et al., 1985; Greuel and Floyd, 1985). The positions of the striated fibers and microtubular rootlets suggest that the organization of the flagellar apparatus in mature cells results from an ~90˚ rotation – in opposite directions – of the two halves of the embryonic flagellar apparatus. This hypothesis is supported by the morphology of cells undergoing flagellar apparatus reorganization (Hoops and Floyd, 1983; Taylor et al., 1985; Greuel and Floyd, 1985).

Since we first observed these changes in flagellar apparatus structure, we have assumed that they were correlated with the change in the orientation of the effective strokes from the opposed pattern exhibited by unicellular volvocalean algae, to the parallel one displayed by the colonial Volvocales (Hoops and Floyd, 1983). Assuming that the flagellar axonemes of the green algae are both structurally and functionally polar – as has been suggested for *Chlamydomonas* (Hoops and Witman, 1983; Hoops et al., 1984) and other algae (Melkonian, 1984) – the flagella would have to adopt a different rotational orientation in the colonial and multicellular species, such as *Volvox*, than they have in the unicellular species in order to generate effective motility. However, until now the only evidence for rotation has come from the altered position of structures external to the basal bodies and flagella, such as the striated fibers and microtubular rootlets. Thus, it has remained formally possible that the basal body/flagellum complex itself does not rotate, but that the change in direction of the effective stroke results from rearrangement of some non-axonemal component. I show here that the axonemes of mature *Volvox* somatic cells do indeed have an altered rotational orientation, and one that is consistent with the postulated rotation of each half of the flagellar apparatus during development, and with the observed flagellar beat patterns.

MATERIALS AND METHODS

Cell culture

Volvox carteri f. *weismannia* (UTEX no. 2180) was obtained from the Culture Collection of Algae at the University of Texas at Austin, and grown in *Volvox* medium as described previously (Hoops, 1984).

Electron microscopy

For the electron microscopic analysis of flagellar polarity, the cells were grown, fixed and embedded between teflon-coated slides as described previously (Hoops, 1984). Spheroids used for study had well-developed gonidia and mature, presenescent somatic cells. In addition, all spheroids analyzed were oriented in a direction that could be unambiguously determined, and were distant enough from neighboring spheroids that any given flagellar cross-section could be unambiguously assigned to the spheroid under study. These selected spheroids were mounted in known orientation on plastic blocks and sectioned onto Formvar-coated slot grids using a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Zeiss 900 TEM equipped with a goniometer stage. Because the tilt necessary to obtain good flagellar cross-sections influenced the magnification, magnifications given here were calculated using known axonemal dimensions rather than the nominal magnification of the microscope.

Analysis of flagellar polarity depends on the ability to correlate flagellar polarity with the orientation of the underlying cell or spheroid. Therefore, precautions were taken to assure that specimens under study were free of unattached flagella, or flagella originating from a second spheroid. Moreover, in many cases, the insertion points of flagella were determined by study of serial sections, and in every case it was as predicted from study of more distal sections.

Preparation of the sample for electron microscopy sometimes resulted in the loss of flagella. If the two flagella had different rotational orientations and one was preferentially lost, it would result in an incorrect average orientation. However, right and left flagella were found to be present in about equal numbers, even in spheroids that were missing many flagella. In addition, it was found that there is little if any difference in orientation between the right and left flagella (see Results). Therefore, it is unlikely that the average flagellar orientation observed here is skewed by overrepresentation of one class of flagella.

The analysis of the flagellar vs cellular vs spheroid orientation required us to determine the spheroid orientation at both the light microscope and ultrastructural levels. Under the growth conditions used here, spheroids usually had 8 gonidia arranged in two tiers of 4 in the posterior. Spheroids selected for study were ones in which the gonidia in a given tier were either overlapping (indicating they were viewed from the side) or oriented in a ring (viewed from the anterior or posterior). Spheroid orientation was confirmed by observing the spacing between cells; cells in the anterior hemisphere are more widely spaced than those in the the posterior.

Cinephotomicrography

Spheroids were placed in a drop of conditioned growth medium on a silicon-coated slide, captured by gentle suction with a micropipet held by a Leitz micromanipulator, and observed with a Zeiss Axioskop equipped with a ×40 water-immersion, phase-contrast objective. Flagellar motility was recorded on Kodak Tri-X 16 mm movie film at 190-290 frames per second with a Redlake Locam model 51 high-speed motion camera synchronized with a Chadwick-Helmuth power supply and strobe.

DIC analysis of cell orientation

To determine the orientation of somatic cells relative to the spheroid, spheroids were fixed with 0.1% glutaraldehyde, lightly compressed between coverslips and scanned for spheroids in which all 4 gonidia from a tier were exactly in line. Such spheroids were photographed under low power to record the polarity of the spheroid and at higher power to determine the orientation of the flagellar tunnels of selected somatic cells, using an American Optical microscope equipped with DIC optics. Pairs of prints were

mounted in the same orientation. Spheroid polarity was determined by drawing a line through the center of the gonidia of both tiers using the lower power micrograph. In principle, these lines are perpendicular to the anterior-posterior axis of the spheroid. If the line connecting the posterior gonidia was not exactly parallel to that connecting the anterior gonidia, the reference line was the average of the two. Cellular polarity was then determined by drawing a line between the two flagellar tunnels of a cell and measuring the angle between this line and the spheroid polarity reference line. An average cellular orientation was determined for each spheroid in which the orientation of at least 7 cells could be determined. Occasionally, cells were observed that had a very different orientation from their neighbors. Such cells were excluded from analysis if they differed from the average cellular orientation by more than 20°.

RESULTS

Terminology of cellular and organismal polarity in *Volvox*

This paper compares the orientation of axonemes relative to the direction of flagellar beat and the orientation of cells at various locations within the spheroid. This involves comparison of orientations at several levels, and potential conflicts in terminology. Therefore, it is necessary to define the terms used. The anterior-posterior axis of the spheroid is defined in the conventional manner, as the axis around which the spheroid rotates, and along which it moves. The anterior pole is defined by the direction of forward motion. In contrast, the anterior-posterior axis of each cell is conventionally defined as extending from the flagellar apparatus (anterior) through the chloroplast (posterior) and is approximately perpendicular to the surface of the spheroid. The cellular axis is seldom the same as the spheroid axis, and the angle between these axes depends on whether the cell is located in the anterior, posterior or side of the spheroid. The right and left flagella are designated as viewed from **inside** the cell, with the anterior pole of the spheroid upward (in other words, from the cell's point of view). These directions are opposite those an outside observer would use when looking down on the spheroid from the outside, and are the opposite of those previously used by Coggin and Kochert (1986) for *V. carteri*. However, they conform to the conventions used by most anatomists to describe orientations in unicellular and multicellular organisms. All cross-sections of basal bodies and flagella, and the graph showing flagellar polarity are printed in the now-conventional manner, that is as if looking from inside the cell outward. The diagrams presented here are drawn in the opposite orientation – as they would appear to an outside observer.

Orientation of the flagellar basal structures with respect to spheroid polarity

In a previous study (Hoops, 1984) I described the structure of the flagellar apparatus of the somatic cells of *V. carteri* and showed that the orientation of the flagellar apparatus of each cell is correlated with its position in the spheroid. Here, to verify and extend that result, I analyzed basal body and rootlet orientation in neighboring cells by thin-section-TEM analysis of spheroids with known orientations. The results are summarized schematically in Fig. 1. In spheroids viewed or sectioned from the side, neighboring cells have nearly the same orientation (Fig. 1A,B). Lines passing through the basal bodies of each cell in a tier are parallel, and at approximately right angles to the anterior-posterior spheroid axis - like lines of latitude on a globe. As previously described, two of the four microtubular rootlets are nearly parallel; these extend toward the spheroid posterior. The other two rootlets are nearly antiparallel, and extend in the same direction as the line connecting sister basal bodies. The distal striated connecting fiber is displaced toward the side of the basal bodies that faces the spheroid anterior. In contrast, cells in sections from near the spheroid poles are arranged differently. Cells near the anterior pole are oriented in such a way that the two inner microtubular rootlets extend outward, away from the center of the section, and the distal striated fibers lie inward, toward the center (Fig. 1C,D). On the other hand, cells near the posterior pole have opposite orientations; their inner rootlets extend towards the center, and their distal fibers lie toward the outer edge of the section being viewed (Fig. 1E,F). Despite these superficial differences in appearance, however, it is clear that cells all around the spheroid are organized according to one simple rule: the nearly parallel microtubular rootlet pairs all point to the spheroid posterior, and the distal fibers are always on the side facing the anterior pole.

The position of the eyespot in relationship to the flagellar apparatus

Because the position of the eyespot has been extensively used as a polarity marker in *Chlamydomonas*, the position of the eyespot in *V. carteri* was determined. The eyespot is located on the side of the cell towards the posterior of the spheroid (Figs 2, 3). The 4-membered rootlet is consistently found over one edge of the eyespot while the other edge comes close to the 2-membered rootlet (Fig. 2).

Flagellar motion and cellular and spheroid polarity

High-speed cinephotomicrographic analysis reveals that the two flagella of *Volvox carteri* f. *weismannia* beat with a ciliary-type motion that resembles that of forward-swimming *Chlamydomonas* in many respects. It is a typical ciliary-type waveform except that, like *Chlamydomonas* (Ringo, 1967; Ruffner and Nultsch, 1985, 1987), there is a partial overlap between the effective and recovery strokes. Like other colonial and multicellular algae examined (Hoops and Floyd, 1983; Taylor et al., 1985), it differs from the beat pattern of *Chlamydomonas* in the following details: first, the effective stroke is directed predominately at right angles to the anterior-posterior axis of the cell in *Volvox*, rather than to the sides as in *Chlamydomonas*. This is presumably correlated with the fact that the basal bodies lie nearly parallel to the anterior-posterior cellular axis in *Volvox*, whereas they are tilted about 45° from that axis in *Chlamydomonas*. Secondly, the two flagella have an effective stroke in nearly the same direction in *Volvox*, whereas in *Chlamydomonas* the effective strokes of the two flagella are initially directed away from one another (Ringo, 1967; Hyams and Borisy, 1978; Ruffner and Nultsch, 1985, 1987). When viewed from the direction of the effective

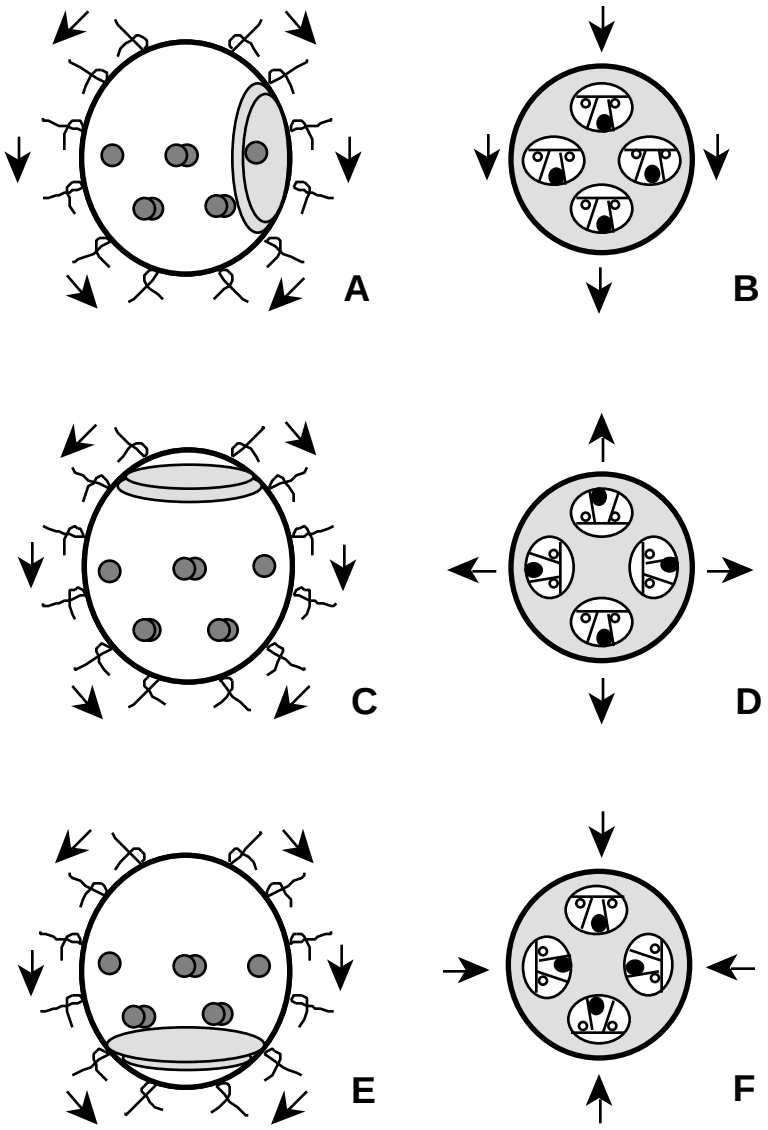

Fig. 1. Relationships between spheroid polarity and somatic cell orientation. In the diagrammatic three-dimensional representations of the spheroids shown to the left (A, C and E), the positions of the gonidia (gray circles) indicate that the anterior pole is toward the top of the page. Arrows indicate the approximate direction of the flagellar effective strokes. The plane of the section to be analyzed is shown in gray. A diagrammatic representation of the organization of nearest-neighbor cells observed in such sections is shown to the right (B, D and F). Each of the sections is oriented as it would appear to an observer outside of the spheroid. Again, the arrows indicate the approximate direction of effective strokes. Within each cellular profile, the orientation of the basal bodies (open circles) microtubular rootlets (continuous lines) and eyespots (filled ovals), are shown. Note that the relative orientation of these structures in neighboring cells is different, depending on whether the section is taken from the side (B), anterior pole (D) or posterior pole (F) of the spheroid. In actuality, prominent eyespots are not found in the spheroid posterior. They are included in F to allow cellular orientation to be compared in different areas of the spheroid and because other markers of cellular orientation are not visible at the resolution of the diagram.

stroke, it is apparent that the flagella beat in nearly parallel planes (Fig. 4). This is confirmed when the flagellar beat is examined from above the spheroid. Such phase-contrast preparations are optically very poor, but in favorable sequences it is possible to determine that the direction of flagellar motion of the two flagella is approximately parallel, with both flagella beating generally towards the spheroid posterior. So far, I have been unsuccessful at precisely determining the direction of the effective stroke because of the difficulty in determining the direction of flagellar motion when filming from the side, and the poor optical quality of the phase-contrast images when viewed from above, but effective strokes directed to the cell's left could account for the counterclockwise rotation displayed by swimming *Volvox*. *Volvox* flagella beating with a flagellar-type waveform have never been observed, in spite of the relatively high light intensity used in filming. No persuasive evidence of any coordination between the flagellar beat cycles of neighboring cells, or even between the two flagella of a single cell has been observed. The ciliary-type motion is not absolutely planar but, like that of *Chlamydomonas* (Ruffner and Nultsch, 1985), has a small three-dimensional component.

Polarity markers in *V. carteri* axonemes

The presence of polarity markers in *C. reinhardtii* (Hoops and Witman, 1983) and in other unicellular green algae (reviewed by Melkonian, 1984) as well as the presence of such a marker in another species of *Volvox* (Melkonian, 1984) suggested that they might also be present in the axonemes of *V. carteri.* Reliable polarity markers were not found in the basal body, transition region or region of the flagellum immediately distal to the transition region. However, every flagellum examined distal to that point had a single doublet that lacked its outer dynein arm (Fig. 5A,B). Occasionally, the doublet lacking an outer dynein arm also possessed a two-part bridge that extended from the A-tubule of the doublet lacking the arm to the B-tubule of the adjacent doublet (Fig. 5B). Also, beak-like projections were occasionally found inside the B-tubules of three doublets (Fig. 5B). When found, these structures were present in the doublet lacking the outer dynein arm and in the two dou-

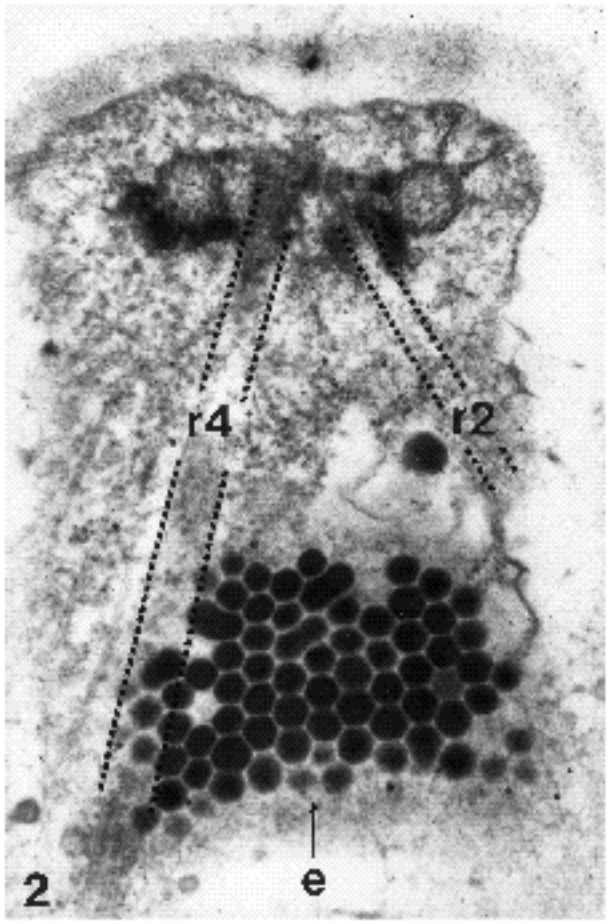

Fig. 2. Eyespot position in *V. carteri.* Note that the 4-membered rootlet (r4) runs near the (cell's) right edge of the eyespot (e). The 2-membered rootlet (r2) lies near the other side of the eyespot. Broken lines indicate the course of the microtubular rootlets determined from the adjacent sections. ×43,000.

blets directly opposite it. These three markers of flagellar polarity are the same as those found in the axonemes of *Chlamydomonas* (Hoops and Witman, 1983). By analogy, the doublet that lacks an outer dynein arm is identified as doublet no. 1; other doublets are numbered 2-9 in a clockwise progression as viewed from inside the cell. There is no consistent relationship between the position of the number 1 doublet and the central pair of microtubules, consistent with the hypothesis that the central pair rotates during the beat cycle, as has been shown in *Chlamydomonas* (Kamiya, 1982; Kamiya et al., 1982).

Does axonemal asymmetry correlate with flagellar apparatus asymmetry?

To answer this question, I sectioned spheroids of known polarity and examined the sections to determine the orientation of the axoneme relative to that of the spheroid and its constitutive cells. This requires that the polarity of flagella and of the spheroid be determined simultaneously. However, sections that revealed the flagellar polarity did not include the usual indicators of spheroid polarity. Therefore, the plane defined by the flagellar bases was used to determine cellular and spheroid polarity. DIC analysis of the flagellar tunnels showed that the flagellar bases were nearly at right angles to the anterior-posterior axis of the spheroid (average orientation is 5.5° counter-clockwise from the cell's perspective; s.d. = 5.0°, n = 23 spheroids).

Dynein arms were usually not present in the axonemes of *V. carteri* while they were still within the flagellar tunnel, and were often absent for at least 10 sections distal to this level. Therefore, it was not possible to determine cellular and flagellar polarity of a particular cell in a single thin section. Instead, neighboring cells were used to determine the approximate cellular polarity. Axonemes having identifiable polarities were identified and photographed along with nearby cells using the same tilt and rotation settings of the goniometer. Useful data could be obtained only when the following three conditions were met: (1) the axoneme under examination showed a clear polarity; (2) the section contained at least one nearby cell with a cross-section of basal bodies or flagellar tunnels so that the axis between them could be unambiguously determined and used to define the orientation of the cell; and (3) the approximate anterior-posterior axis of the spheroid could be established by virtue of the positions of eyespots, rootlets or other flagellar apparatus structures. In addition, the same axoneme on adjacent sections was usually examined to confirm the identification of axonemal polarity.

Polarity of axonemes in spheroids sectioned from the side

An example of the application of these methods to determine axonemal and cellular polarity is shown in Fig. 6. The spheroid has been oriented such that the flagellar bases run horizontally (Fig. 6A). The position of the eyespot and the microtubular rootlets of the cell in Fig. 6B indicate that the posterior pole of the spheroid is down. The two flagella analyzed for polarity are seen at the top of Fig. 6B and at higher magnification, but with the same orientation, in Fig. 6C. Their position indicates that they are probably the right and left flagella of a single cell that was located slightly below this plane of section. In both of these flagella, the number 1 doublet faces the anterior of the spheroid. That this is a reproducible result can be seen in Fig. 7. Each of the flagella in this gallery has been arranged with the same orientation as the flagella in Fig. 6 (that is, with the anterior pole of the spheroid up, and the axis between flagellar bases horizontal). The number one doublets are all located on the side of the flagella toward the spheroid anterior.

Polarity of axonemes from spheroids sectioned near anterior and posterior poles

If the rotational orientation of the flagella is correlated with cellular and spheroid polarity rather than the plane of section, then the polarity of the flagella in the anterior and posterior of the spheroid would be expected to differ from those of cells on the side, in accordance with the results summarized diagrammatically in Fig. 1D and F. Flagella of anterior cells beat away from each other, and when axonemes of such flagella are analyzed, the number one doublet faces *toward* the center of the section (Fig. 8A,B). In contrast, in the cells from the posterior, where the flagella beat toward one another, the number one doublets face *away* from the center of the section (Fig. 8C,D).

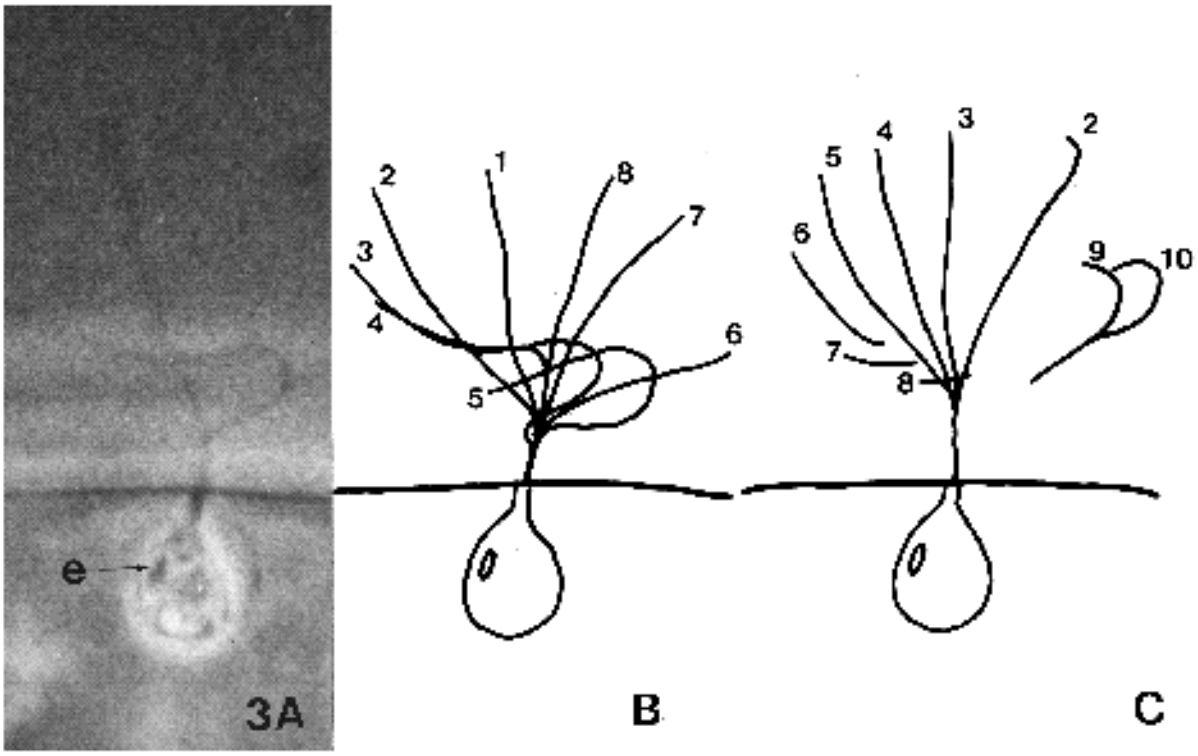

Fig. 3. Flagellar motion in *V. carteri* filmed at 190 frames per second. (A) Phase-contrast image of a cell taken from frame number 4. (B,C). Tracings of flagellar beat patterns for both flagella of the cell shown in A. The numbers identify the frame number for this sequence. Both flagella can be seen beating in approximately the same direction and towards the posterior of the spheroid. Note also that the flagella beat towards the portion of the cell that contains the eyespot (e). ×1300.

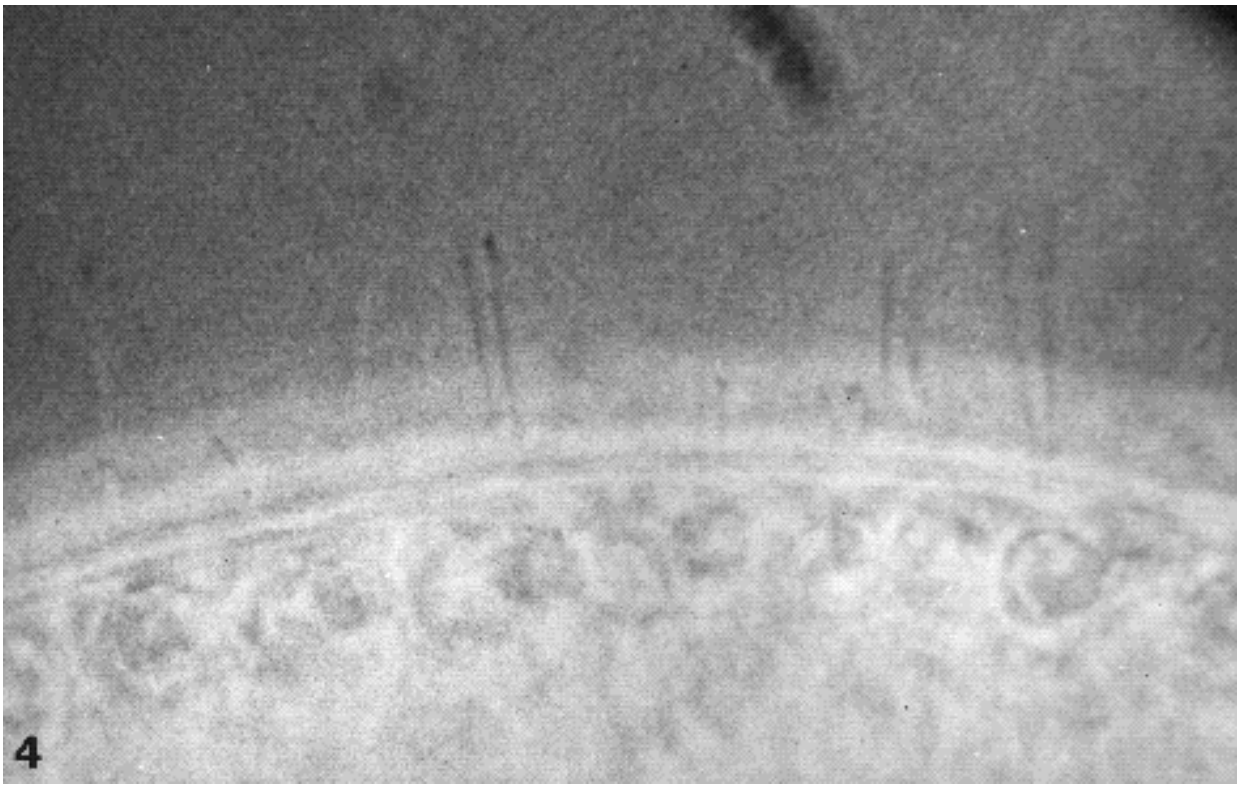

Fig. 4. Flagellar motion filmed at an orientation at right angles to that in Fig. 3. Here the spheroid is being held so that it's anterior-posterior axis runs towards the observer. The two flagella of each cell are nearly parallel through the effective and recovery strokes. ×1300.

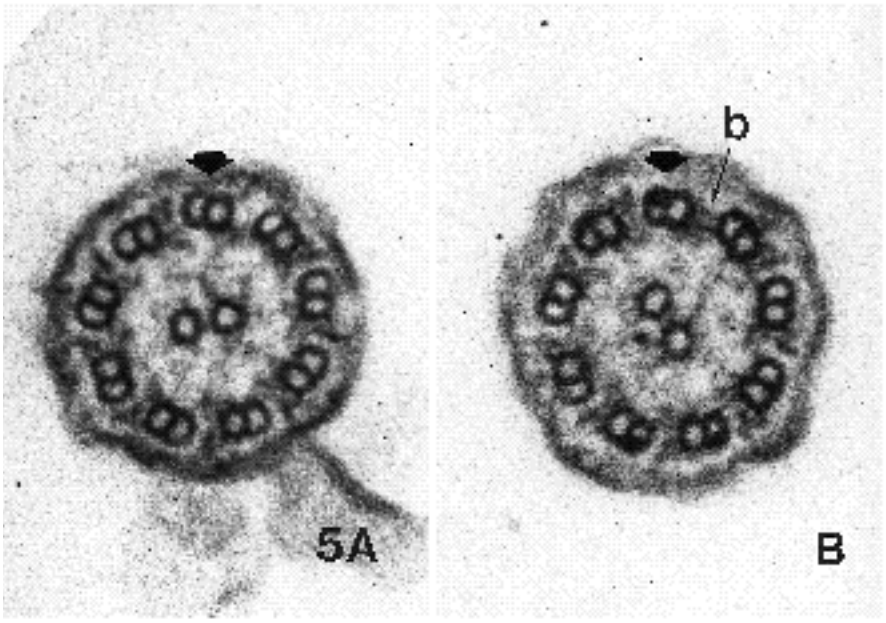

Fig. 5. Cross-sections showing polarity markers in *Volvox carteri* flagella. The doublet lacking the outer dynein arm (the number 1 doublet) is marked by the arrowhead. (A) The most frequently observed form of axonemal asymmetry, in which only the absence of one dynein arm is seen. (B) Occasionally, two additional markers of asymmetry are seen – a two part bridge (b) can be seen going from doublet number 1 to doublet number 2 and beak-like projections in the B-tubules of doublets 1, 5 and 6. ×180,000.

In short, cells in all parts of the spheroid have axonemes that are oriented such that the number 1 doublet is approximately perpendicular to the axis between the flagella and on the side toward the spheroid anterior. This also means, of course, that in each case the number 1 doublet is on the side of the axoneme approximately opposite the side toward which the effective stroke would have been directed in life.

Quantification of axonemal polarity

To quantify axonemal polarity, I determined the position of the number 1 doublet relative to the nearest cell(s) that showed the orientation of the flagellar bases. A line was drawn through the center of the flagellum parallel to the line connecting the flagellar bases of the nearest reference cell and then the angle of the number 1 doublet was determined (to the nearest 5˚) relative to the normal of the reference line.

There are two potential problems that would prevent an accurate assessment of the polarity determined in this way. First, this method uses the polarity of one cell to determine the polarity of a neighboring cell that is out of the plane of section. Because the cells are on the three-dimensional surface of the sphere, neighboring cells must have slightly different polarities. In this respect, the analysis runs into the same problem faced by map makers trying to represent the three-dimensional globe on a two-dimensional surface. To minimize these effects, the cells used to determine polarity of the basal structures were chosen to be as close as possible to those of the flagella being analyzed. Secondly, if the flagellar axoneme displayed any twist, the apparent polarity of the flagella would be different at different distances from the spheroid surface. If the axoneme was twisted severely enough for this to have an effect over the relatively short distance between the spheroid surface and the level of the flagellum cross-section analyzed, it should be apparent; no evidence of such twist was found. Nevertheless, it is conceivable that such twists might make a minor contribution to the scatter of the data.

Overall axonemal orientation

Thirty one axonemes from the anterior, posterior and side of the spheroid were analyzed in this way (Fig. 9). Of these, 29 had the number 1 doublet at nearly right angles to the line connecting the flagellar bases, with a mean value of 5.6˚ clockwise from the cell's perspective (s.d. = 18.1˚). Two points lay outside of this cluster, but although they had a significant effect on the standard deviation, they did not have any effect on the mean orientation (average including all points = 5.6˚; s.d. = 31.8˚). The significance of these outlying points is not known. However, occasionally a few cells in a spheroid have abnormal cellular orientations; such cells probably have flagella with correspondingly altered axonemal orientations.

Is the orientation of the right and left flagella different?

Because *Volvox* normally has a very ordered cell arrangement, it is generally possible to infer the positions of the underlying cells, and hence identify left and right flagella even when the section containing the flagella may not include any portion of the cell body from which they emanated. The orientation of flagella tentatively identified as right flagella vs left flagella was compared, to determine if the two differed significantly in their orientations (Fig. 9). The average orientation of the right flagellum (+3˚; s.d. = 17.2˚; n = 10) was not significantly different from that of the left flagellum (+8˚; s.d. = 16.2˚; n = 10).

DISCUSSION

Axonemal polarity markers

The flagella of *V. carteri* contain axonemal-polarity markers similar to those of the unicellular *C. reinhardtii.* This is not surprising, considering the presumed close evolutionary relationship between these two species (Crow, 1918; Roberts, 1974; Adair et al., 1987; Goodenough and Heuser,

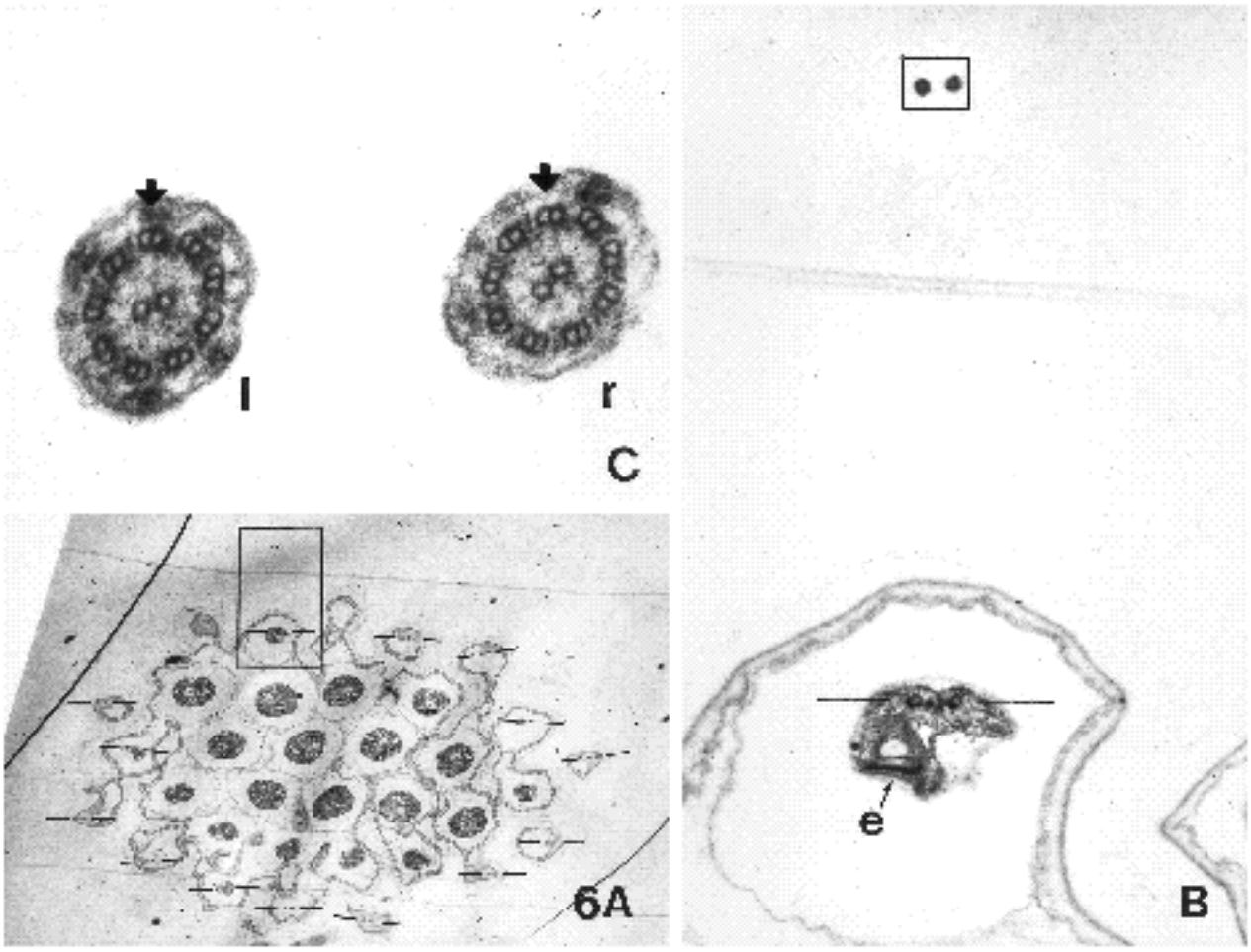

Fig. 6. Analysis of rotational orientation of flagella from cells at the side of the spheroid. (A) Low magnification view of a section through the entire spheroid. Note that the lines connecting the flagellar tunnels of each cell are nearly parallel. ×1,200. (B) An enlargement of the boxed area of A showing a cell that can be used to determine the orientation of the cells relative to that of the spheroid. The cell is sectioned through both basal bodies, indicating that the anterior-posterior axis is very close to vertical. The eyespot (e) is located towards the spheroid posterior from the basal bodies, therefore the spheroid posterior is towards the bottom of the page. Two flagella (boxed) can be seen in positions suggesting that they have arisen from a single cell immediately out of the plane of the section. ×8,400. (C) An enlargement of these two flagella showing their polarity. The print is mounted in the same orientation as A and B. In both flagella, the number 1 doublet (arrow) faces towards the top of the page (the spheroid anterior), and about 90˚ from the line that connects the two basal bodies. The presumptive left (l) and right (r) flagella are labeled. ×105,000.

1988; Matsuda, 1988; Buchheim and Chapman, 1991; Larson et al., 1992). Somewhat more surprising is the fact that the flagella of *V. carteri* only rarely show the two-part bridge or beak-like projections that are regular features of the axonemes of *Chlamydomonas.* In view of the presence of beak-like structures in algae evolutionarily more distant from *Chlamydomonas* (Hoops et al., 1982), and their occasional presence in *V. carteri,* they might be expected to be regular features of the *V. carteri* axoneme. It is possible that they are regularly present in the flagella of living *V. carteri,* but are seldom preserved under the fixation methods used in this study. Alternatively, the difference might have a functional basis. In *Chlamydomonas* the beak-like projections have been postulated to be associated with the ability to execute both the ciliary-type waveform used by forward-swimming cells, and the flagellar-type waveform used by backward-swimming cells (Segal et al., 1984). Curiously, however, whereas flagella of a *Chlamydomonas* mutant that lacks the normal complement of beak-like projections beat only with the flagellar-type waveform, their apparent absence in *Volvox* is correlated with flagella that only undergo ciliary-type motion.

Unlike the case in *Chlamydomonas,* the number 1 doublets of the two axonemes do not face each other in mature *V. carteri* cells; rather, both point in the same direction, and perpendicular to a line connecting them. This implies that the two axonemes have each been rotated by about 90˚ but in opposite directions during development.

Cellular asymmetry

The *Chlamydomonas* cell, like other unicellular volvocalean algae, at first glance appears to be symmetrical. However, detailed analysis of the flagellar apparatus, eyespot, contractile vacuoles, mating structure and mitotic poles reveals a fundamental cellular asymmetry that appears to be intimately associated with behavior of the basal bodies during

mitosis (Holmes and Dutcher, 1989). This cellular asymmetry is demonstrated by the positions of cellular structures associated with particular microtubular rootlets (notably the eyespot and mating structure; Goodenough and Weiss, 1978; Huang et al., 1982) and by developmental (Huang et al., 1982; Melkonian et al., 1987) and functional differences in the basal body and flagellum (Kamiya and Witman, 1984; Ruffner and Nultsch, 1985, 1987). In *Volvox*, as in other colonial and multicellular members of the order, the cellular asymmetry is much more prominent (Pocock, 1933; Gerisch, 1959; Coggen and Kochert, 1986). *Volvox* contains the same markers of cellular asymmetry as the uni-

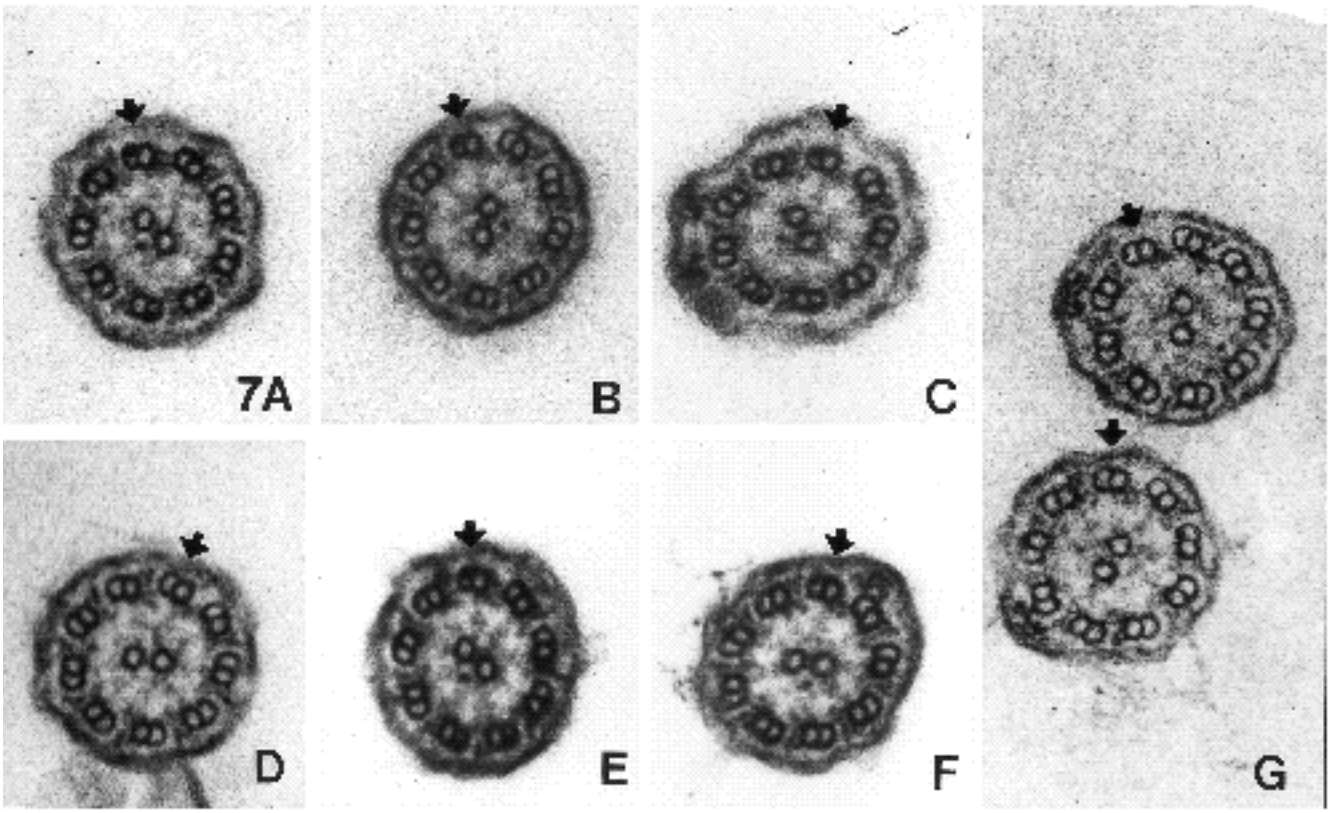

Fig. 7. A gallery of flagellar orientations from cells sectioned from the side of the spheroid. In each case the images are mounted such that the anterior pole of the spheroid is toward the top of the page, and a line connecting the flagellar bases is horizontal. The number 1 doublets are indicated by arrows. A, C and F are presumptive right flagella and D is presumed to be a left flagellum. C and D are from the same cell. ×125,000.

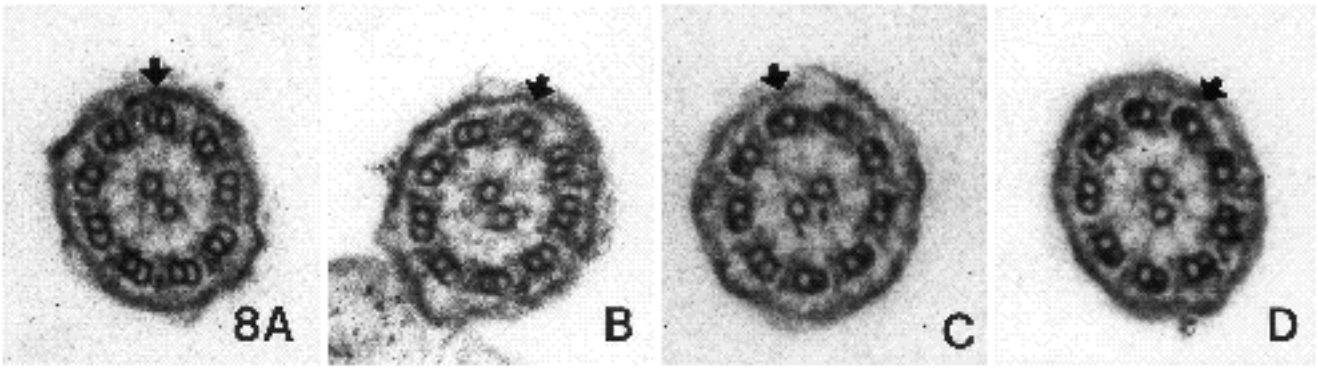

Fig. 8. Flagellar polarity in cells from the colonial anterior and colonial posterior. The number 1 doublet is indicated in each case by an arrow. The prints are mounted so that the line connecting two sister flagellar bases is horizontal, and the inferred anterior pole is toward the top of the page. (A,B) Polarity of flagella from cells near the anterior pole. The center of the section is towards the top of the page. A is probably from a left flagellum. (C,D) Flagellar polarity from cells near the posterior pole. The center of the section is towards the bottom of the page. C is from a presumed left flagellum and D is the presumed right flagellum from the same cell. ×125,000.

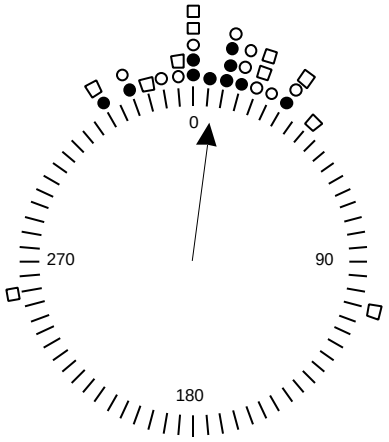

Fig. 9. Distribution of the rotational orientations of flagella. The measurements represent the orientation of the number 1 doublet as measured from the perpendicular to a line passing between the flagellar bases. The graph is oriented with the anterior pole of spheroid towards the top of the page and from the cell's perspective (i.e. a clockwise rotation is to the cell's right). The central vector represents the mean orientation of all flagella; it is not changed appreciably when the two outlying points are omitted. Data points which come from left or right flagella are indicated by filled or open circles, respectively. Points which come from flagella that cannot be assigned to one of these categories are shown as open squares.

cellular forms, but their positions are changed during maturation of the flagellar apparatus. The demonstration that the basal bodies do indeed rotate confirms our suggestion that the flagellar apparatus of the colonial and multicellular Volvocales results from rotation of each half of the flagellar apparatus. It is therefore necessary to take rotation of the basal bodies and associated structures into account before they can be reliably used to compare cellular polarity in these two forms.

On the basis of the observation that the eyespot and the mating structures are found in specific locations with respect to particular flagellar rootlets (Goodenough and Weiss, 1978; Holmes and Dutcher, 1989), Holmes and Dutcher, (1989) have suggested that the cells of *Chlamydomonas* can be divided by a plane perpendicular to that connecting the basal bodies into *cis* and *trans* halves, and by a second plane passing through the basal bodies into *syn* and *anti* halves. These names are assigned with respect to the position of the eyespot, which is always located in the *syn*/*cis* quadrant (Fig. 10A). Despite the differences in basal-apparatus morphology that results from rotation, it is possible to apply these same axes to the *Volvox* cell, which has secondarily lost the superficial symmetry shown by *Chlamydomonas.* In *Chlamydomonas* and other algae, the eyespot lies on one side of one of the larger (4-membered) microtubular rootlets (Gruber and Rosario, 1974; Moestrup, 1978; Goodenough and Weiss, 1978; Melkonian and Robenek, 1984; Holmes and Dutcher, 1989). In mature somatic cells of *Volvox,* the eyespot is located between one 4-membered and one 2-membered microtubular rootlet. Presumably, during reorganization of the basal apparatus, the eyespot and the 4-membered microtubule rootlet with which it is associated have rotated in the counter-clockwise

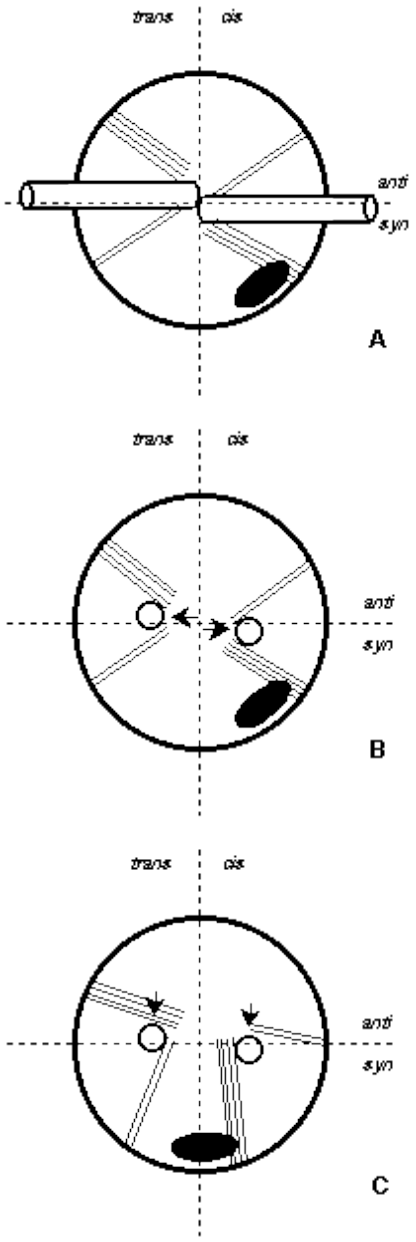

Fig. 10. Comparison of flagellar and cellular asymmetry in *Chlamydomonas* and *Volvox.* (A) Cellular and flagellar polarity in *Chlamydomonas* after Hoops and Witman (1983) and Holmes and Dutcher (1989). Terminology is after the latter authors. The view is from outside the cell. The number 1 axonemal doublets run along the anterior edges of both flagella (i.e. facing the viewer). Striated fibers are omitted for clarity. The immature flagellar apparatus of *V. carteri* has a similar overall distribution of basal bodies, striated fibers and microtubular rootlets (Hoops, 1984). (B) As above, except that the basal bodies and flagella extend parallel to the anterior-posterior cell axis and toward the viewer. The number one doublets (arrows) now face each other. (C) Orientation in mature somatic cells of *Volvox carteri.* Each half of the flagellar apparatus has been rotated, as can be seen by the positions of the microtubular rootlets (Hoops, 1984) and the position of the number 1 doublets (this study). Note that the eyespot (filled oval) has moved with the 4-membered rootlet, and now lies close to the *cis-trans* axis; however, the 4-membered rootlet with which it is associated still defines the *cis/syn* quadrant of the cell.

direction (from the cell's perspective) while one 2-membered microtubule rootlet emanating from the other basal body was rotating clockwise, until it came to lie near the other edge of the eyespot. The *V. carteri* somatic cell can also be divided into *syn*/*anti* and *cis*/*trans* halves by planes passing through and between the basal bodies, respectively (Fig. 10). However, because of this rotational displacement, the eyespot is bisected by the *cis*/*trans* plane and does not, by itself, define the *cis*/*trans* plane as it does in *Chlamydomonas* and related unicellular algae (Huang et al., 1982; Holmes and Dutcher, 1989). I propose that the terms *syn* and *trans* may still be used to indicate cell polarity in cases where the original position of the eyespot can be inferred. In *V. carteri* somatic cells the *syn*/*cis* quadrant can be defined not by the eyespot per se, but by the 4-membered microtubular rootlet associated with it (Fig. 10C).

Regardless of the position of the somatic cells within the spheroid, the *anti* side always faces the anterior pole of the spheroid and the *syn* side faces the posterior pole. In addition the *trans* side is on the cell's right and the *cis* side is on its left.

Rotation of the basal apparatus

In *Volvox*, the plane of flagellar motion and the orientations of the two axonemes are both rotated by a similar amount relative to their orientations in *Chlamydomonas*. This suggests that there is an intrinsic structural polarity of the axoneme that is related to the functional polarity of the flagellar beat in both *Chlamydomonas* and *Volvox*. Presumably the need for effective swimming has supplied the selective pressure that has resulted in the rather dramatic reorganization of the flagellar basal structures in the colonial and multicellular Volvocales. This also suggests that the flagellar apparatus modifications probably occurred early in the evolution of the colonial green flagellates.

The axonemal orientations reported here agree with the earlier analyses of flagellar development in *V. carteri* and *V. rousseletii* (Hoops, 1984), *Platydorina caudata* (Taylor et al., 1985), *Astrephomene gubernaculifera* (Hoops and Floyd, 1983) and *Gonium pectorale* (Greuel and Floyd, 1985). In each of these cases, the flagellar apparatus of the cells during and immediately after cleavage was observed to be similar to that of *Chlamydomonas*. In all of these cases (save the four central-most cells of *Gonium*), however, the flagellar apparatus lost 180˚ rotational symmetry as the cells differentiated and the flagellar apparatus matured. This structural modification has been interpreted to be the result of rotation of each half of the basal apparatus. The present demonstration that the number 1 doublets face the same direction in *Volvox*, rather than opposite directions as in algae such as *Chlamydomonas*, strongly supports this hypothesis. The altered axonemal orientation suggests that the *cis* half of the flagellar apparatus rotates counterclockwise (from the cell's perspective) while the *trans* half rotates clockwise. The cellular mechanism that causes this rotation is completely unknown.

The sites at which the microtubular rootlets and the distal fiber are attached to the basal bodies also rotate during the reorientation of the basal body/flagellum complex. This suggests that these appendages are always attached to a particular set of basal body triplets. We had previously suggested that these fibers may set up the proper rotational positioning of the basal body/flagellar complex by binding to particular points on the surface of the basal body in *Chlamydomonas* (Hoops and Witman, 1983; Hoops et al., 1984). The flagellar apparatus of cleaving volvocalean cells is very similar to that of *Chlamydomonas*, so similar mechanisms may be involved in forming the immature flagellar apparatus of *Volvox*. Because rotation of the basal bodies during flagellar apparatus maturation occurs after this stage, it requires a reorganization of the entire flagellar apparatus.

Basal body rotation is, however, not unique to colonial and multicellular algae such as *Volvox*. In all biflagellate green algae, basal bodies are replicated and transmitted semi-conservatively. Thus, after division a new flagellum grows from a basal body that matured during the last division cycle and each pair of basal bodies includes one new and one old one (reviewed by Melkonian et al., 1987; Segaar and Gerritsen, 1989). If the absolute orientation of the flagellar apparatus is to be preserved, then it is essential that in every division cycle there be rotation of the old basal body with respect to the newer one (O'Kelly and Floyd, 1984). Such rotation has been observed in *Chlamydomonas* by Holmes and Dutcher (1989) and in the related unicell *Brachiomonas* by Segaar and Gerritsen (1989). If the schemes proposed by Holmes and Dutcher, and Segaar and Gerritsen, are correct for the division stages of *Volvox* – and there is no reason to think that they are not – the older (*trans*) basal body of each pair must rotate counterclockwise (as viewed from the cell's perspective) in each division cycle. Thus, this basal body rotates in opposite directions in the two episodes of basal body rotation. Segaar and Gerritsen (1989) suggested that sliding of oppositely oriented 4-membered rootlets may be responsible for the rotation of the basal bodies in *Brachiomonas* after mitotic division. In *Volvox*, such a mechanism might be involved in the first episode of rotation (to set up the *Chlamydomonas*-type flagellar apparatus typical of immature cells), but it cannot be responsible for the later rotation, because the two 4-membered rootlets in the flagellar apparatus do not overlap one another during this period.

Spheroid motility

I have shown that both flagella of the *V. carteri* cell beat in nearly parallel planes toward the posterior pole. This would account for the forward motion of the spheroid, but not for its rotation. However, effective strokes directed to the cell's left (the spheroid's right as viewed from the exterior) would result in the observed ('left-hand screw') rotation. For technical reasons, I was unable to measure the precise angle between the flagellar effective strokes and the anterior-posterior axis of the spheroid using phase-contrast microscopy, and so cannot confirm this assumption. We are now using DIC optical sectioning of flagellar beat as viewed from above to answer this question.

The analysis of axonemal orientation did not give evidence for effective strokes directed at an angle to the spheroid axis. The mean values for the tilt of the basal body pair (5.5˚ counterclockwise, from the cell's perspective) and the orientation of the number one doublet (5.6˚ clockwise), would result in the number 1 doublets facing directly toward the spheroid anterior. If these values are correct, and

if the flagella beat directly away from the number one doublet and towards doublets number 5 and 6, as we proposed for *Chlamydomonas* (Hoops and Witman, 1983), then flagellar orientation does not account for the observed spheroid rotation. It is possible that the number 1 doublet of one or both axonemes is actually rotated clockwise from the mean values obtained in this study. However, it is more likely that the direction of the effective stroke may not be exactly towards the number 5 and 6 doublets in *Volvox*. We had originally determined that the number 1 doublet of each *Chlamydomonas* axoneme directly faced the second, and the two flagella are known to beat away from one another at the start of the effective stroke (Ringo, 1967; Hyams and Borisy, 1978; Ruffner and Nultsch, 1985, 1987). Our analysis of flagellar orientation and motility assumed that the two flagella beat precisely in the plane of the basal bodies. However, observations by Ruffner and Nultsch (1985) suggest that this might not be correct. Therefore, it is possible that the flagella do not beat directly towards the number 5 and 6 doublets even in *Chlamydomonas.*

If the direction of the effective strokes are structurally determined, and if the flagellar orientation is specified as a result of the cell's structural organization and polarity, then the orientation of the cell within the spheroid will determine the orientation of flagellar motion. Analysis of cellular orientations indicates that the spheroid possesses rotational symmetry. In the context of the *Volvox* spheroid, rotational symmetry can be operationally defined by the ability to rotate any sector from the plane perpendicular to the anterior-posterior axis around a central point with resultant overlap of homologous structures. Because each somatic cell has a *cis* side and a *trans* side, overlap of cellular structures can only be produced by rotation and not by reflection. This excludes the use of the term 'radial symmetry' which, in biology, usually describes objects as having multiple lines of mirror image symmetry. That the spheroid exhibits rotational symmetry is very obvious in the anterior and posterior tiers (see Fig. 1), but this symmetry is also characteristic of the other tiers. Rotational symmetry is the simplest cellular arrangement that results in spheroid rotation during forward swimming. Rotational symmetry of the flagellated cells within the spheroid will ensure that any lateral component of flagellar motion will contribute to rotation of the spheroid, as long as all cells in the spheroid behave similarly (e.g., that a component of the effective stroke of one or both flagella is directed to each cell's left).

This symmetry is probably formed during the first cleavages. After three cleavages, the embryo of another forma of *V. carteri* (Starr, 1969; Green and Kirk, 1981, 1982) like many other colonial and multicellular volvocalean algae (Pocock, 1953; Stein, 1958; Gerisch, 1959; Goldstein, 1964; Nozaki, 1986) form the 'Volvox cross' (Gerisch, 1959) with a prominent 90˚ rotational symmetry. The rotational symmetry of cell positions is preserved through subsequent cleavage events, as illustrated with exceptional clarity in the SEM images of developing *V. carteri* (Green and Kirk, 1981, 1982). However, the development and maintenance of the cellular orientation must be a little more complex. Huskey (1979) has isolated a number of mutants in which the cells have random cellular orientations as determined by the position of the flagellar tunnels and the eyespot. As one might expect, these spheroids do not display effective motility. However, the cells are found in their normal position in the spheroid. This organization reflects a precise set of cleavages in the *Volvox* embryo (Starr, 1969; Green and Kirk, 1981, 1982). It would seem unlikely that these cleavages could be maintained without the correct polarity of the embryonic cells. This suggests that the cells developed their random orientation after the completion of cleavage, but before the individual cell walls and their associated flagellar tunnels were laid down. The mechanism by which the cells develop their proper orientation within the colony is unknown, but the process may require functional flagella (Huskey, 1979).

I thank Kim Feathers for her assistance in high-speed cinephotomicrography and David Kirk for comments on the manuscript. This work was supported by NSF grants 895078 and BSR-9020013.

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(Received 30 June 1992 - Accepted 9 September 1992)