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Mirror-imaged doublets of *Tetmemena pustulata*: Implications for the development of left–right asymmetry

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Abstract

Ciliated protozoa possess cellular axes reflected in the arrangement of their ciliature. Upon transverse fission, daughter cells develop an identical ciliary pattern, ensuring perpetuation of the cellular phenotype. Experimentally manipulated cells can be induced to form atypical phenotypes, capable of intraclonal propagation and regeneration after encystment. One such phenotype in the ciliate *Tetmemena pustulata* (formerly *Stylonychia pustulata*) is the mirror-imaged doublet. These cells possess two distinct sets of ciliature, juxtaposed on the surfaces in mirror image symmetry, with a common anterior–posterior axis. We have examined whether individual ciliary components of *Tetmemena* mirror-image doublets are mirror imaged. Ultrastructural analysis indicates that despite global mirror imaging of the ciliature, detailed organization of the membranelles is reversed in the mirror-image oral apparatus (OA), such that the ciliary effective stroke propels food away from the OA. Assembly of compound ciliary structures of both OAs starts out identically, but as the structures associated with the mirror-image OA continue to form, the new set of membranelles undergoes a 180° planar rotation on the ventral surface relative to the same structures in the typical OA. The overall symmetry of the OA thus appears to be separable from the more localized assembly of individual basal bodies. True mirror imagery of the membranelles would require new enantiomorphic forms of the individual ciliary components, particularly the basal bodies, which is never observed. These observations suggest a mechanistic hypothesis with implications for the development of left–right asymmetry not only in ciliates, but perhaps also in development of left–right asymmetry in general.

Keywords: Cortical inheritance; Cilia; Spirotrichs; Global patterning; Local assembly

Introduction

Spirotrichs (formerly hypotrichs) possess different types of compound ciliary structures arranged in a highly asymmetric and polarized fashion on the cell surface, with each of these structures possessing its own inherent asymmetry and polarity. These ciliary structures develop in a predictable and reproducible manner. When these cells undergo division or reorganization, nascent cortical primordia form, which become or replace the functional structures found in fully differentiated cells. Primordia arise from disaggregation of pre-existing ciliary structures and directed assembly of basal bodies adjacent to, and sometimes away from, existing basal bodies (Grimes, 1973a). Primordia formation follows the same developmental sequence regardless of the type of the event taking place in the cell, whether division, reorganization, regeneration or excystment processes (Grimes, 1989). In certain spirotrichs, all ciliary structures and associated basal bodies are completely resorbed during cyst formation, but reform completely upon excystment (Grimes, 1973c,d). This implies that some marker components must be left in the cortex to identify the sites of ciliary organelle assembly and alignment upon excystment. Mechanisms of cortical inheritance in protozoa have remained an intriguing mystery since the work of Sonneborn (1963, 1964).

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Extra sets of ciliary structures can be acquired and maintained on the cortices of a clonal cell line for years after generation of the progenitor cell. These supernumerary ciliary structures can also reappear after a sequence of encystment and excystment. Stable phenotypic variants that divide true to type have been generated in spirotrichs by techniques such as microsurgery, thermal shock and chemical shock. These variants include a homopolar doublet first described by Dawson (1920), a subtype of this phenotype described by Grimes (1973b) and a "humped cell" phenotype generated by Grimes (1976) using a microbeam laser.

The phenotype used in this study is the mirror-image doublet (MID), which is also referred to as buccal-opposed mirror-image doublets by Shi and Frankel (Shi et al., 1991). This phenotype was first observed by E. Fauré-Fremiet (1945) and later described by Tchang et al. (1964). MIDs have approximately twice the number of ventral cortical structures, juxtaposed and arranged in mirror imaged fashion. The two halves share a common polar anterior/posterior axis, but the lateral (left/right) axes of the two halves are reversed. Tchang et al. (1964) and Totwen-Nowakowska (1965) first generated MIDs in *Stylonychia mytilus*. Subsequently, MIDs were generated in other spirotrichs such as *S. mytilus* (Grimes et al., 1980; Shi and Qiu, 1989; Tuffrau and Totwen-Nowakowska, 1988), *Pleurotricha lanceolata* (Grimes et al., 1980), *Paraurostyla weissei* (Jerka-Dziadosz, 1983) and *Tetmemena pustulata* (Yano and Suhama, 1991). Examination of encysted cells at electron microscopic resolution reveals no evident differentiated cortical structures (Grimes, 1973c). As in other instances, the MID in *Tetmemena* persists upon excystment and is asexually inherited for many generations. Results from conjugation experiments (Shi and Qiu, 1989) suggest that the mirror-image doublet genotype is identical to the singlet genotype. These results imply that nuclear genes play little or no role in determining assembly and orientation of membranelles and undulating membranes of the right mirror-imaged OA in a doublet, but simply code for proteins used to construct these cortical structures. Once the progenitor structures are generated for a particular phenotype, the existing cortical structures themselves seem to ensure the propagation of the global pattern (Grimes and Aufderheide, 1991).

The focus of this study is a detailed examination of the ultrastructure of individual ciliary components of the two sets of oral structures of a mirror-image doublet in *Tetmemena* to determine their intrinsic asymmetries. The results have important functional and developmental implications pertinent to the interaction of information systems that determine phenotypic morphology, global patterning and assembly and positioning of cell structures. These implications might not pertain exclusively to spirotrichs, but also potentially for vertebrate development, particularly because persistent inheritance of ciliated structures seems largely orchestrated by non-genic directed assembly events. One significant conclusion is that although basal bodies behave as if affected by a left–right gradient during cortical morphogenesis in the mirror-image doublet, the invariance of basal body enantiomorphism destroys true mirror image symmetry.

Materials and methods

Organism

Cells used in this study were isolated by one of us (Grimes) in Northport Long Island, NY. Their anatomy and development correspond exactly to *T. pustulata* (*Stylonychia pustulata*), a species that is very similar to *Sterkiella*, both in its morphology and morphogenesis (Eigner, 1999) and its molecular signature (Foissner et al., 2004; Hewitt et al., 2003).

Cell culture techniques and microscopy of living cells

Singlet cells of *T. pustulata* were isolated and cultured at room temperature with *Tetrahymena* and zooflagellates as prey in spring water supplemented with wheat seed. Mirror-image doublets derived from these singlet cells were cultured in the same manner. Living cells were observed under a dissection microscope or placed in a rotary compression chamber manufactured for live observation of ciliary beat in a bright field/phase contrast microscope.

Generation of mirror-image doublet phenotype

The mirror-image doublet phenotype was obtained by heat shocking small numbers of singlet cells during the S phase of the cell cycle. Depression wells, containing cells in macronuclear S phase, were floated on the surface of a water bath (55 °C) for 20 s. Shocked cells were placed in fresh culture medium and allowed to regenerate overnight. Abnormal cells were isolated and cultured. Phenotypes were identified by light microscopy and verified by SEM. The convention used here for describing the ventral surface of *Tetmemena* is from the inside of the cell looking out (i.e. the cells' aspect). The axes shown in all micrographs reflect this convention; left and right arrows correspond to the left and right sides of a cell. Most cells studied were morphostatic (i.e. not in some stage of reorganization or division) but some cells were examined during oral morphogenesis.

Scanning electron microscopy (SEM)

Cells were fixed for 20 min at room temperature in a 2:1 mixture of 2% osmium tetroxide and 2% glutaraldehyde, buffered with 0.1 M sodium cacodylate (pH 6.9). After a single 10-min buffer wash with sodium cacodylate, cells were dehydrated in an increasing ethanol series and infiltrated with Freon 113 prior to critical-point drying in liquid CO_2. Cells were mounted individually onto stubs with rubber cement (Elmers Products, Inc., Columbus, OH), coated with gold-palladium (60:40) alloy and imaged with a Hitachi S-2460N SEM operated at 20 kV.

Transmission electron microscopy (TEM)

With the exception of three sodium cacodylate buffer washes following initial fixation, preservation of cells for TEM was identical to the fixation protocol used for SEM. After dehydration with an ethanol series, cells were infiltrated with propylene oxide, flat embedded in Epon 812 resin and polymerized in a vacuum oven at 60 °C for 48 h. Individual cells were cut out and mounted on blank resin blocks using "Krazy Glue" (Borden Inc., Columbus, OH) for microtomy. Thin (~75 nm) sections were taken with a diamond knife and adhered to formvar-coated slot grids (1 mm×2 mm). Sections were post-stained with saturated aqueous uranyl acetate for 20 min followed by Reynolds' (1963) lead citrate for 10 s. A Philips EM-201 TEM operated at 60 kV was used to view and record sections on Kodak 4489 film.

Results

Cortical organization of Tetmemena

The stichotrich species (see Lynn and Small, 2005, for taxonomy) used in this study is approximately 150 μm in

length, dorso-ventrally flattened with an asymmetrical arrangement of cortical structures (Figs. 1a, c). Stichotrich species have definitive lateral (left/right), polar (anterior/posterior) and dorsal/ventral axes due to the asymmetric arrangement of ciliary structures on the cortex of the cell. This phenotype is seen in cell culture without experimental manipulation and represents the wild type cell.

The oral apparatus (OA) of this organism is composed of an adoral zone of membranelles (AZM; Fig. 1a) and two undulating membranes (um; Figs. 2a, b) plus an inconspicuous cytostome (cell mouth) located near the posterior end of the AZM. The resulting ciliary structure is highly asymmetric, possessing distinct lateral and polar axes. Ventral cirri (fc, mvc, tc; Fig. 1a) are composed of hexagonally packed ciliated basal bodies. These cirri are ultrastructurally very similar to one another, differing only with respect to number of basal bodies comprising each structure. Marginal cirri, located at the left and right margins of the cell, are similar to the cirrus types described above (rmc and lmc; Fig. 1a). However, the basal bodies of marginal cirri are arranged in a rectangular rather than polygonal fashion and are composed of fewer basal bodies. The dorsal surface of spirotrich ciliates has 6 rows of short cilia called dorsal bristles (db; Fig. 1c) plus 3 caudal cirri at the posterior end.

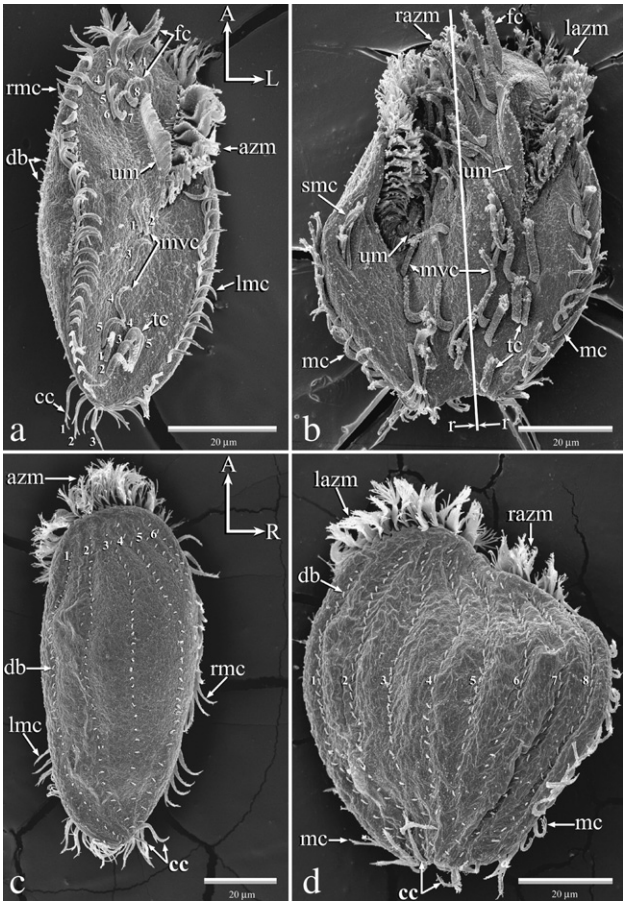


Fig. 1. SEM of ventral and dorsal surfaces of singlet (a, c) and mirror-image doublet (b, d) cells. Perpendicular arrows indicate polarity and asymmetry of the cell from the cells perspective; anterior (A), left (L), right (R). Scale bars equal 20 µm. (a) Ventral surface of *Tetmemena* singlet cell. Frontal cirri (fc) 1–8, midventral cirri (mvc) 1–5, transverse cirri (tc) 1–5, left marginal cirri (lmc), right marginal cirri (rmc) and caudal cirri 1–3 (cc). The OA is comprised of adoral zone membranelles (azm) and undulating membranes (um). (b) Ventral surface of a *Tetmemena* mirror-image doublet cell. The cell possesses approximately double the number of frontal, midventral and transverse cirri of a singlet cell. In addition, two OAs ventrally juxtaposed and arranged in mirror-image fashion, can be seen at the anterior left (lazm and um) and right (razm and um) margins of the cell. The vertical line indicates the approximate axis of bilateral symmetry of the cell, with lateral arrows indicating the perceived fused right (r) axes of each half of the cell. Supernumerary marginal cirri (smc) are present on the mirror image (razm) side; marginal cirri (mc). (c) Dorsal surface of a singlet cell. Six longitudinal rows of dorsal bristles (db) are arranged on the dorsal surface. Rows 1–4 extend the length of the cell unlike rows 5 and 6. Each dorsal bristle unit is composed of one ciliated and one nonciliated basal body. (d) Dorsal surface of a mirror-image doublet cell. Typically, 8 rows of dorsal bristles extend the length of the cell. Rows 5 and 6 are not present on either of the two halves of the cell; contractile vacuole pore (cv).

Several ancillary structures are associated with the basal bodies in *Tetmemena*. Postciliary microtubules (MTs) extend from some basal bodies of ciliary structures on the ventral surface (pc; Figs. 2b, c, e, f). Those associated with the most posterior row of any given ciliary structure are directed toward the posterior of the cell. Kinetodesmal fibers composed of filamentous subunits are located to the anatomical right of the postciliary MTs and are also directed posteriorly from the posterior portion of a basal body. Microfibrillar networks extend between basal bodies of individual ciliary structures to link basal bodies.

Cortical organization of mirror-image doublets

Oral apparatus

Mirror-image doublets of *Tetmemena* have two sets of cortical structures juxtaposed on the same surface sharing a common cytoplasm, resulting in cells that possess two sets of ventral and dorsal ciliary structures sharing common ventral and dorsal surfaces (Figs. 1b, d). The two sets of cortical structures have similar polar axes but distinct lateral axes, yielding a left and right mirror-image cortex (Fig. 1b). The *left OA* (*typical OA*) is ultrastructurally indistinguishable from the OA of a typical singlet cell. The *right OA* (*mirror-image OA*) is arranged as a global mirror image of the left OA on the ventral surface of the cell, such that if folded along the longitudinal axis at the midline, the organelles would overlap with reasonable precision (Fig. 1b). This indicates that anterior–posterior differentiation of the apparatus is unaffected, whereas overall left–right axis is reversed in the right half of the cell. The UMs of the right OA and the UMs of the left OA are juxtaposed with cirri separating the two sets. Membranelles of the right OA encompass the anterior right portion of the cell and extend posteriorly at an oblique angle to the right margin toward the midline of the cell. However, ultrastructural analysis of the right OA of mirror-image doublets reveals that while left and right sets of oral structures are mirror imaged globally, they are not mirror images locally. Membranelles of the right OA are 180° planarly rotated relative to membranelles of the left OA (Figs. 2d and 3c). Although row 3 of each membranelle is juxtaposed to the left margin of the membranelle, as would be expected in the mirror-image OA, row 3 is now posterior to rows 1 and 2 and row 4 is posterior to row 3.

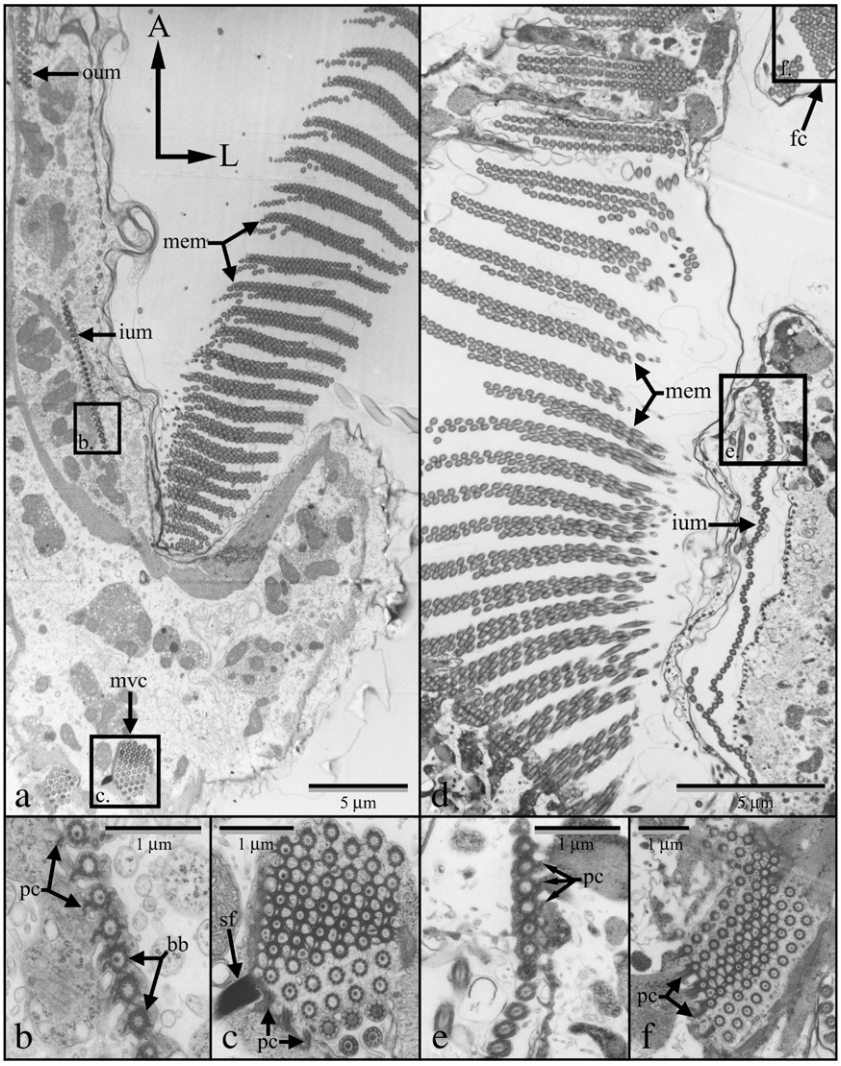


Fig. 2. TEM of typical vs. mirror-image OAs of *Tetmemena.* (a–c) The membranelles (mem), inner undulating membrane (ium) and outer undulating membrane (oum) are shown. The ium is to the anatomical right of the membranelles. The shorter membranelle row 3 is anterior to rows 1 and 2. Mid-ventral cirri (mvc) are seen posterior to the oral region. Scale bar equals 5 µm. (b) High magnification of UM in Fig. 2a (boxed region). Postciliary microtubules (pc) are oriented toward the posterior of the cell; basal body (bb). Scale bar equals 1 µm. (c) High magnification of a mid-ventral cirrus in panel a. Postciliary microtubules (pc) of basal bodies (bb) are oriented toward the posterior of the cell. A striated fiber (sf) is associated with the anatomical right of the cirrus. Scale bar equals 1 µm. (d–f) Corresponding images of a mirror-image OA from a MID. (d) The ium is located to the anatomical left of the membranelles. The shorter row 3 is posterior to rows 1 and 2. A frontal cirrus (fc) can be seen in the top right corner of the micrograph. Scale bar equals 5 µm. (e) High magnification of UM in panel d. Orientation of postciliary microtubules is identical to orientation seen in singlet cells. Scale bar equals 1 µm. (f) High magnification of frontal cirrus in panel d through basal body region. Postciliary microtubules are oriented toward the posterior of the cell. Scale bar equals 1 µm.

Basal bodies

The membranelle rows are composed of aligned ciliary basal bodies. As elsewhere, the basal bodies are radially symmetric with asymmetric organization of internal components, which gives them a particular directionality or enantiomorphic form. In both left and right OA membranelles, basal bodies invariably have a counterclockwise "rotational" orientation when viewed from the outside of the cell looking in, which is reflected in the position of subfiber A relative to subfiber B in the nine ciliary axonemal doublets. A consequence of invariant enantiomorphic form of the basal bodies is disruption of true mirror imaging of the membranelles during morphogenesis, such that the right OA forms by 180° planar rotation of invariantly assembled basal bodies. This produces a situation whereby postciliary microtubules and kinetodesmal fibers associated with basal bodies of membranelles in the right OA are oriented toward the anterior (Fig. 3c) instead of the posterior, as seen in the left OA (Fig. 3b).

Intrinsic left–right axis of cilia determines beat direction

Fig. 3a (insert) shows a 9+2 ciliary cross-section of a typical membranelle of the left OA at high magnification. In this section, the central pair of microtubules is oriented approximately 90° to the plane of the ciliary row (doublet no. 1 is marked with an asterisk). A cilium cross-section is diagrammed in Fig. 4a #5, where an axis perpendicular to the central pair is indicated and the doublets are numbered. By convention, the axis passes through doublet number 1. The position of the postciliary MTs (pc, Fig. 3a) corresponds to the

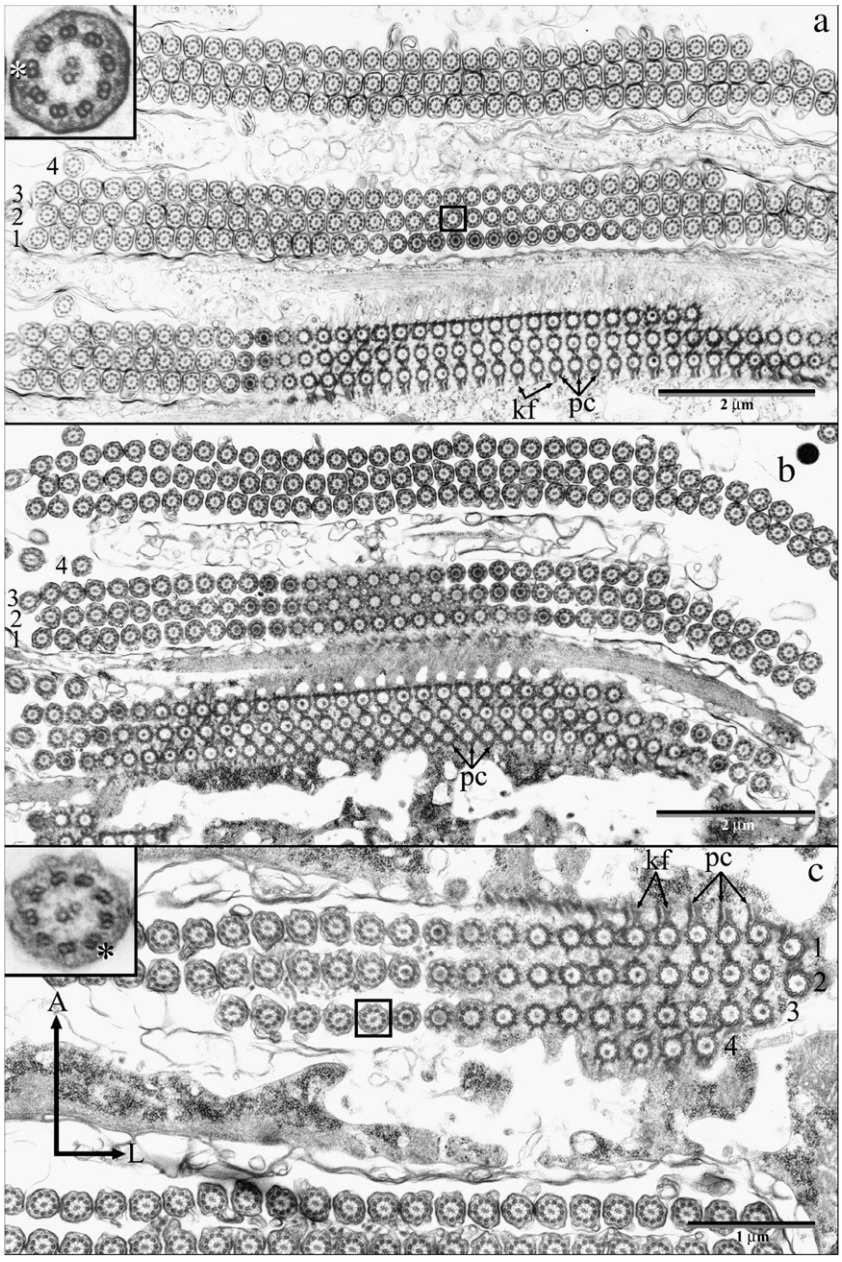
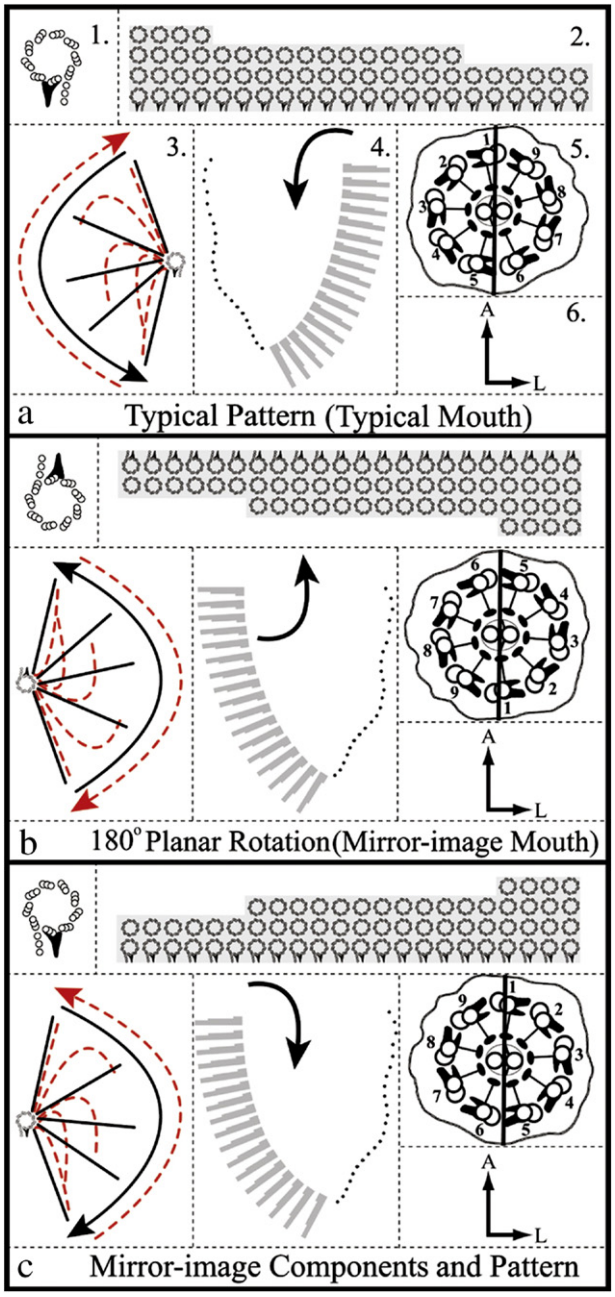


Fig. 3. Comparative membranelle cross sections of (a) a typical singlet, (b) a typical OA of a mirror-image doublet and (c) a mirror-image OA of a mirror-image doublet. Perpendicular arrows in panel c indicate polarity and asymmetry of the membranelles in all three micrographs; anterior (A), left (L). (a) Singlet cell. The cross-section at the top of the micrograph is through cilia, whereas the cross-section at the bottom is through corresponding basal bodies. In each membranelle, basal body row 1 is posteriormost, whereas row 4 is anteriormost. Kinetodesmal fibers (kf) and postciliary microtubules (pc) are oriented toward the posterior of the cell. The kinetodesmal fiber is located to the anatomical right (viewer's left) of the postciliary microtubules. Scale bar equals 2 µm. Inset: High magnification of boxed cilium. Asterisk denotes microtubule doublet pair number 1. (b) Typical OA of an MID. Orientations of rows, kinetodesmal fibers and postciliary microtubules are identical to those seen in a typical singlet cell. Scale bar equals 2 µm. (c) Mirror-image OA of a MID. These membranelles are 180° planarly rotated membranelles shown in panels a and b. Row 4 is posteriormost and row 1 is anteriormost. The kinetodesmal fibers and postciliary microtubules are oriented toward the anterior. Scale bar equals 1 µm. Inset: High magnification of boxed cilium. Asterisk denotes microtubule doublet pair number 1.

direction of the effective stroke. Because the dynein arms responsible for doublet sliding and motility all function as minus-end motors, opposite sides of the axoneme (switching dynein activity) are responsible for generating the effective and recovery strokes (Satir, 1985). In these axonemes, as is usual in other situations, the effective stroke (Fig. 4a #3 solid arrow) is toward doublets 5–6 and depends on dynein activity of doublets 1–4, whereas the recovery stroke (Fig. 4a #3 dashed arrow) depends on the activity of doublets 6–9 (Satir and Matsuoka, 1989). The left (typical) OA anatomical axes are diagrammed in Fig. 4a #6. Doublets 2–4 define the axoneme's right side; their dynein arms extend in the effective stroke direction, here toward the posterior of the cell, whereas doublets 7–9 define the axoneme's left side; here anterior. The effective stroke of the cilia in Fig. 3a will push food posteriorly into the cytostome.

Fig. 3c (insert) shows a corresponding cross-section from a right OA membranelle. The central pair lies approximately in the membranelle's plane. A cross-section of this cilium is diagrammed in Fig. 4b #5. Because the dynein arms are still oriented in the clockwise direction, doublets 7–9 still define the left side of the axoneme, even though the effective left–right axis of the OA is reversed. To achieve this orientation, doublet 1 must now lie at the posterior side of the axoneme. Correspondingly, the postciliary microtubules (pc, Fig. 3c) lie anteriorly and the effective stroke (dependent on the activity of doublets 1–4) will now push food anteriorly, out of the cytostome, so that the right OA alone is not able to provide sufficient nourishment for cell maintenance (Grimes, 1989).

The direction of ciliary beat has been observed directly in mirror-image doublets. The left membranelle band creates a flow that directs particles into the left OA cytostome, whereas particles and water are swept away from the right OA cytostome in the mirror image half of the cell. Ultrastructural evidence (not shown) suggests that membrane recycling occurs at and near the cytostome of the mirror image OA. In protozoa, membranes for food vacuole formation are transported from the cytoplasm via microtubules to the cytostomal region of the OA (Allen, 1974), which suggests that the cytostomal region of the mirror-image OA would be capable of forming food vacuoles if food were directed toward it.



Cortical morphogenesis of Tetmemena

Morphogenesis of the typical OA of *T. pustulata* and related species has been described previously (Grimes, 1972; Grimes and Adler, 1976; Wirnsberger et al., 1985, 1986). This description also applies to the development of a new left OA in the MIDs described here. Prefission morphogenesis begins in mid macronuclear S phase with the appearance of a small patch of basal bodies (kinetosomes) in an amorphic field close to the left margin of transverse cirrus number 5 below the existing OA. Membranelle formation proceeds with the pairing of basal bodies to form couplets (Fig. 5a). Basal bodies assemble *in situ* and couplets align to the right side of each primordial membranelle with doublets 6–9 of each basal body oriented to the left as in Fig. 4a. Additional couplets are added to the left side of existing aligned couplets to form rows #1 and #2 of each membranelle. Microfibers connect to adjacent couplets to form a microfibrillar network between basal bodies. The number of kinetosomes gradually increases and the field extends anteriorly until it is positioned just posterior to the existing OA and to the left of the ventral cirri. The field continues to elongate and widen at the anterior end as kinetosomes continue to align on the right side. The oldest, most mature membranelles of the new AZM are anteriormost in the new left OA. Kinetosomes from

Fig. 4. Diagrams of membranelle and ciliary configurations. Basal body and membranelle orientations as well as cilia beat forms and membranelle patterns of the OA are shown for three different OAs. Diagrams of ciliary cross-sections with doublet pairs 1–9 numbered are shown (5). Cellular polarity and asymmetry are indicated (6); anterior (A), left (L). (a) The typical pattern seen in singlet cells and the left side of mirror-image cells. The basal body (1) is arranged as a right pinwheel (as seen from the outside of the cell looking in). The membranelles (2) are oriented such that the shorter third and fourth rows are most anterior. The anteriormost membranelles of the OA mature first. The effective stroke (solid black arrow, 3) of the cilia in the membranelles is directed posteriorly (with a counterclockwise rotation) and the recovery stroke (dashed arrow, 3) is directed anteriorly. Food is directed toward the cytostome of the OA (thick arrow, 4). (b) The pattern seen in a mirror-image OA. Basal bodies are still arranged as a right pinwheel; however, the third and fourth rows are now posteriormost. As a result of this planar rotation, the posteriormost membranelles mature first while the effective stroke of the cilia is directed anteriorly and the recovery stroke posteriorly. Food is expelled from the OA. (c) True mirror imaging of components and pattern of membranelles. Complete mirror imaging of the membranelles would require assembly of mirror-image basal bodies (left pinwheel), leading to assembly of mirror-image membranelles, which is not observed. Mirror imaging of the basal bodies would result in a clockwise rotation of the cilia with the effective stroke directed posteriorly and the recovery stroke directed anteriorly, thereby directing food into the cytostome.

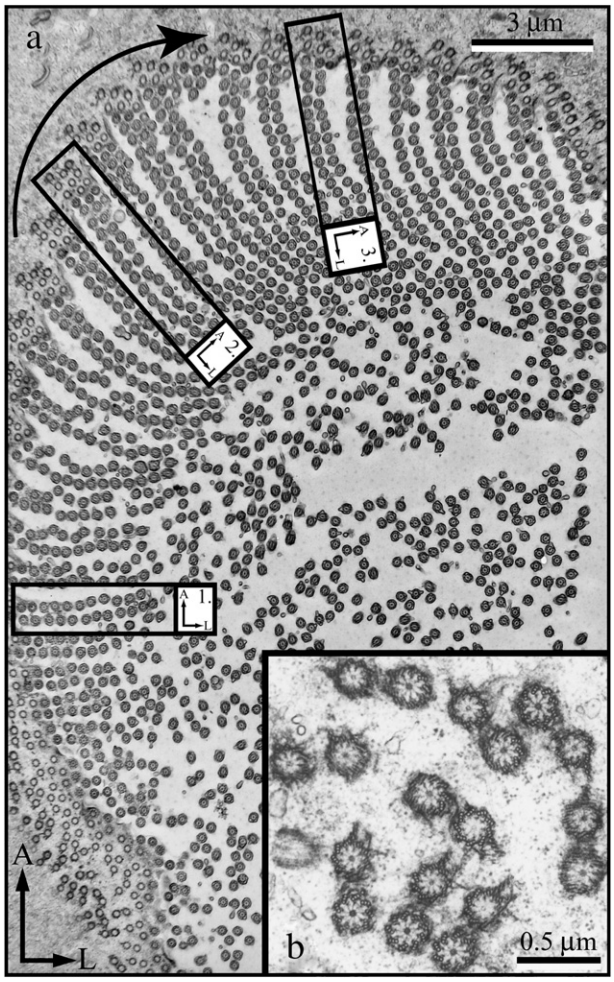


Fig. 5. Oral primordium of mirror-image half of mirror-image doublet. (a) Basal bodies can be seen pairing up in the bottom left hand side of micrograph. Rows become increasingly longer toward the anterior of the cell. As elongation proceeds, the membranelle rows begin to turn toward the left (viewer's right) margin of the cell (curved arrow), which is never observed in the left half (typical side) of a mirror-image doublet. Rectangles #1–3 indicate orientation of individual membranelles relative to cell axes. The orientation of rectangle #1 corresponds to the typical membranelle orientation that is retained in the typical OA of the MID, whereas rectangles #2 and #3 indicate a counter-clockwise (viewer's clockwise) rotation of the membranelles relative to the cell axes. Cell axes are indicated at the bottom left hand corner; anterior (A), left (L). Scale bar equals 3 µm. (b) High magnification of basal body pairing taken from the lower left region just below the frame of panel a. Scale bar equals 0.5 µm.

the anterior-right margin of the forming OA migrate from the anterior-right quadrant to form a primordial field for the new UM and frontal cirrus number 1 for the new cell. Basal bodies also form *in situ*, anterior and to the right side of row #2, to form rows #3 and #4 of each membranelle. As membranelles align, new cirri develop from disaggregation of existing cirri and the developing oral primordium. Remaining ciliature is resorbed.

Marginal cirral primordia are the last of the ventral primordia to develop and do so within existing rows of marginal cirri as the other primordia continue to differentiate. Dorsal bristle (DB) primordia for rows 1–3 differentiate from within existing rows to become the new DB rows for the mother and daughter cells. Furthermore, the primordium from DB row 3 separates into two segments to form DB rows 3 and 4. Primordia for dorsal bristle rows 5 and 6 are formed from the anterior regions of both right marginal cirri primordia. Three caudal cirri differentiate at the posterior ends of three of the new dorsal ciliary rows (cc; Figs. 1a, c, d) (Grimes and Adler, 1976). The result of this developmental sequence is duplication of the original global pattern from a series of local assembly events resulting in the formation of a second cell or cortical reorganization of an existing cell (Grimes, 1989).

Morphogenesis of the mirror-image right OA does not proceed in mirror image fashion. Instead, in the early stage of development, the right OA primordium begins to assemble in the same manner as the left OA primordium. Basal body couplets begin to link together (Fig. 5b) and form rows 1 and 2 of each membranelle, from right to left in the usual way (Fig. 5a). After these two rows attain a certain length, row 3 begins to form at the anterior right margin of row 2. Now, however, as membranelle assembly continues, the primordium begins to rotate counterclockwise (Fig. 5a, curved arrow) toward the right margin of the cell and as development continues, the primordium eventually completes a 180° planar rotation. In this way, basal body enantiomorphism remains intact, but the basal bodies are rotated to the position seen in Fig. 4b. However, in the developing right mouth primordium, the oldest membranelles are not the anterior membranelles of the AZM, as would be the case in a true mirror image, but are those that assemble first and end up posteriormost when rotation is complete (Fig. 5a).

Undulating membranes

Mirror imaging affects the undulating membranes in the same manner as the membranelles. The left OA UMs of a MID are identical to those of a singlet cell, both in placement on the cell cortex and ultrastructure of the basal bodies. The postciliary microtubules associated with the basal bodies of the inner UM of this OA are oriented toward the posterior of the cell (Fig. 2d).

The right OA UMs are organized in mirror image to the left OA UMs. However, the postciliary microtubules associated with the basal bodies of the right OA inner UM are oriented toward the anterior of the cell (Fig. 2e). This observation was also made in MID cells of *P. weissei* (Jerka-Dziadosz, 1983). As in the membranelles of the right OA, the failure to produce true mirror image symmetry of the basal bodies leads to a reversed and physiologically futile direction of the ciliary effective stroke. Postciliary microtubules were not examined for the outer UMs. Discontinuities can often be observed along the length of the UM in the mirror-image OA (Fig. 2d).

Ventral and marginal cirri

In contrast to the OA, mirror imaging affects the positioning, but not the left–right symmetry, of the cirri. The ventral cirri associated with the mirror-imaged right half of the cell are ultrastructurally identical to those on the left side of a mirror-image doublet, which in turn are identical to those found in a typical singlet cell (Figs. 2c, f). Rootlet fiber associations are not mirror imaged on the right side of a mirror-image doublet. In

addition, the postciliary MTs are oriented toward the posterior of the cell in all ventral cirri. Correspondingly, the ciliary beat of the cirri is unchanged on the right side of a MID.

As with the ventral cirri, no structural differences were observed between marginal cirri associated with opposite sides of a mirror-image doublet cell and those associated with a typical singlet cell. Postciliary microtubule orientation appears to be identical in cirri on both halves of the mirror-image doublet and corresponds to the orientation found in a typical singlet cell. The mirror-image side, however, typically has supernumerary rows of marginal cirri, which vary in number and length. Some cells possess only one short supernumerary row (Fig. 1b), whereas other cells possess up to one complete and three incomplete supernumerary rows, as described previously for other spirotrichs (Jerka-Dziadosz, 1985; Shi and Frankel, 1990). Shi and Frankel (1990) reported that mirror-image doublets (of the variety described here) of *Stylonychia* possess only left-marginal cirri type. That finding has not been confirmed in this study but has important implications in the development of MIDs, which is discussed in more detail below.

Dorsal bristles

The number of dorsal bristle rows in mirror-image doublets varies from 7 to 10 but the majority of cells examined in this study possessed 8 rows that extended the entire length of the cells. Interestingly, none of the cells examined possessed rows 5 and 6, the short rows associated with the right side of singlet cells. Presumably, this is due to the fact that rows 5 and 6 are derived from primordial fields derived from the right marginal cirri, which these cells apparently do not possess.

Discussion

Global patterning vs. local assembly

The asymmetrical body form of a typical ciliate has a distinct anterior/posterior axis, lateral axis and dorsal/ventral axis with cortical structures arranged in a particular pattern with respect to these three mutually perpendicular axes. The processes that bring about this overall arrangement of organelles have been called "global patterning". Another informational system present in cells, which is responsible for the structuring of individual organelles, has been referred to as local assembly (Grimes et al., 1980). Assembly of individual structures, such as basal bodies, is independent and invariant, dependent upon protein manufacture and self-assembly conditions. These two interactive yet distinct informational systems work in a hierarchical manner such that assembly of organelles is independent of global influence, but positioning of the assembled organelles in the cortex is influenced by the global patterning system (Aufderheide et al., 1980; Grimes, 1989). Some principles of interaction of these two systems can be demonstrated by experimentally manipulating infraciliature such as inverted rows in *Paramecium* (Beisson and Sonneborn, 1965) or the mirror-image doublets in *Tetmemena* studied here.

Mirror-image doublets in spirotrichs

In the mirror-image doublet, the OAs are arranged as mirror images of one another on a global scale, but due to constraints in local assembly as a consequence of the inviolate enantiomorphic assembly of the basal bodies, true mirror imagery of the membranelles and UM cannot be achieved. Instead, a quasi mirror image is achieved where the oral structures of the right OA are rotated 180° relative to the oral structures of the left OA. The consequences of this rotation were first documented by light microscopy of protargol-impregnated specimens of the stichotrich *P. lanceolata* (Grimes et al., 1980), and later confirmed ultrastructurally (in *P. weissei*) by Jerka-Dziadosz (1983). Rotation of the mirror-image OA during development was first described by Shi and Frankel (1990) in protargol stained specimens. We have confirmed those results here at the EM level. Because of this 180° rotation, the power stroke of the cilia of the membranelles in the right OA is directed anteriorly, effectively sweeping food away from, rather than into the mouth (Grimes, 1989). Fortunately, the left OA functions properly, enabling the stable propagation of this phenotype in optimal culturing conditions.

Mirror-image doublets of the type described here have been generated in at least four genera of stichotrich ciliates in six labs over the last 40 years. Such a mirror-image doublet divides true to type and is thus a stable phenotype. In addition, the mirror-image doublet phenotype is stable throughout the cyst stage of the life cycle (Grimes and Hammersmith, 1980). Thus, like the homopolar doublet produced and studied by Grimes (1973b), the global and local patterning of a mirror-image doublet can be sustained in the absence of visible ciliature (Grimes, 1989).

The cells used in this study were generated by thermal shock. The same results have been achieved by microsurgical disruption of the ciliary pattern (Grimes et al., 1980; Shi et al., 1991; Tchang et al., 1964) or by abortive conjugation (Jerka-Dziadosz, 1983; Tuffrau and Totwen-Nowakowska, 1988). None of these techniques or occurrences are considered to be mutagenic in nature (i.e. nucleic acid sequences are almost certainly unchanged). Phenotypic stability depends on cortical inheritance of the type discussed by Beisson and Sonneborn (1965) and others, and is not a result of changes in the nuclear genome. When mirror-image doublets of *Tetmemena* are subjected to physiological stress (i.e. poor water quality, overcrowding, etc.) that does not produce encystment, they convert to the singlet phenotype by absorbing or amputating the right half of the cell. Singlet cells derived from mirror-image doublets maintain the singlet phenotype even after being transferred into an optimal culture environment. When mirror-image doublets are cultured in optimal conditions, however, the phenotype can be maintained indefinitely.

Mechanistic hypothesis for left–right asymmetry generation in mirror-image doublets

Little progress has been made in determining the molecular mechanism of cortical inheritance in protozoa since the original observations of the phenomenon by Sonneborn and colleagues (Beisson and Sonneborn, 1965; Frankel, 1989). Because such

mechanisms might involve common features of organelle assembly and positioning with respect to the cell axis, the mechanisms could prove more generally applicable to metazoan and particularly vertebrate developmental processes. In 1991, Shi et al. (1991) proposed the intercalary reorganization hypothesis, which states that an abnormal juxtaposition of distant regions of the cell is necessary and sufficient for the reversal of anteroposterior axis. This hypothesis is based on the presumption that particular regions of the cell are positionally nonequivalent (Lewis and Wolpert, 1976). When regions of the cell are placed in abnormal juxtaposition to one another, the cell intercalates these regions by the shortest permissible route (French et al., 1976; Mittenthal, 1981). According to the intercalary reorganization hypothesis, the reversal of anteroposterior axis of an OA appears to be due to the abnormal juxtapositioning of right or left marginal cirri. This is an important observation because if MIDs possess only left marginal cirri on a single cell, this could mean that the marginal cirri are critical cortical markers for correct positioning of the basal bodies comprising the oral apparatus during OA development. However, this hypothesis, while descriptively predictive, fails to elucidate a molecular mechanism behind MID reversal.

Clearly some information, probably in the form of a protein or a small protein complex, must be present in the cortex to serve as a marker for basal body placement. This is evidenced by the fact that no obvious structures corresponding to basal body assembly sites are visible by TEM performed on cysts of *Oxytricha fallax* (Grimes, 1973d). This protein or complex must persist and retain its spatial organization through cystment processes and posses its own inherent asymmetry. This hypothesis is supported by research done in *P. weissei* (Fleury et al., 1993), which indicates that "pericentriolar material" is responsible for basal body assembly at particular locations on the cell cortex. A monoclonal antibody (CTR210) made against metazoan centrosomes appears to label the pericentriolar material surrounding basal bodies. When this antibody is used to label *P. weissei* cells in the zygocyst stage of development, "tracks" of pericentriolar material are labeled, which demarcate regions of future basal body assembly. Unfortunately, the specific component of the pericentriolar material that this antibody is recognizing remains unknown. However, this experiment does provide support for the existence of a protein or protein complex that is responsible for the placement of basal bodies in the cortices of ciliated protozoa. Furthermore, this protein/protein complex could possess its own inherent asymmetry and prion-like properties of persistence that basal bodies utilize for proper alignment in the cortex, which allows for the possibility that rotation of this protein/protein complex in the cortex is at least partially responsible for anteroposterior reversal of the OA in mirror-image doublets. For the purposes of this paper, we will refer to this hypothetical protein/protein complex as the Organizing Principle (protein) of the Cortex (OPC).

Basal bodies know left from right

Despite the global mirror imaging shown in Fig. 1b, when examined in detail from the aspect of the membranelle (Fig. 3b vs. c), mirror imagery is incomplete. We suggest that this is a consequence of the unique enantiomorphism of the basal body. The 9+2 ciliary axoneme clearly has "left" and "right" sides, which determine the direction of the recovery and effective strokes, respectively. The basal body from which the axoneme grows has been considered to have only radial symmetry (Fulton, 1971). However, this is probably not correct, since basal body projections, such as kinetodesmal fibers or postciliary microtubules, lie in specific positions (i.e. the rule of desmodexy (Chatton and Lwoff, 1935b)) with regard to basal body triplets. This suggests that, just as in the axoneme, basal body triplets might be specifically defined and given a number (1–9) that corresponds to the doublet number in the axoneme.

Basal bodies (and centrioles) are self-assembled organelles comprised of tubulins, some quite specific, tektins and other less well characterized constituents (Dutcher, 2003). Their production probably relies on coordinated gene expression and has properties similar to viral self-assembly (Satir et al., 2007). The self-assembly process is strictly controlled to produce only one enantiomorphic form of the organelle. Looking inward from the distal end adjacent to the cell membrane, the nine triplets are always assembled with the A, B and C microtubule components following one another in a clockwise fashion, resulting in a counterclockwise pinwheel. The basal body always assembles with triplets 7–9 defining its left side and triplets 2–4 defining its right side. We postulate that basal bodies are then positioned in the cell cortex by the interaction of a specific side of the basal body with the OPC, such that the basal body is positioned to correctly direct effective ciliary beat, which is necessary for cell survival. Parenthetically, a similar interaction must occur in nodal cilia to determine left–right symmetry in the vertebrate body, since all nodal cilia must beat in the same direction (Hirokawa et al., 2006; Nonaka et al., 1998). The effective stroke of these cilia creates a fluid flow only from right to left sides of the node (Hirokawa et al., 2006) because every basal body on the nodal cells is aligned in the same way with respect to the axis of the node. Nodal basal bodies, like the basal bodies of spirotrichs, know left from right, and this becomes reflected first by gene activation patterns and then in organ morphogenesis. Another example is in tetrapod respiratory epithelial cells which have several hundred cilia aligned in rows. These develop in an unaligned manner in a fibrogranular center similar to a protistan anarchic field, but then align so that they all have their effective strokes (and doublets 5–6) in the same direction, eventually to move mucus toward the pharynx (Dirksen and Crocker, 1966). Mutations that affect alignment produce primary ciliary dyskinesia (PCD) (De Iongh and Rutland, 1989; Rautiainen et al., 1990). We would hypothesize that orthologs of the OPC exist in vertebrate cells and that a corresponding interaction between the OPC and basal bodies is involved in the positioning of both motile and primary cilia on the vertebrate cell surface. The latter is especially interesting because of the suggestion that the primary cilium (and the nodal cilium) acts as a cellular GPS (global positioning system) (Benzing and Walz, 2006; Christensen and Ott, 2007), which would apply in gradient sensing, in the control of cell migration direction during development and in

left–right asymmetry determination. Because the cilium is a conserved organelle that evolved early in the history of eukaryotes (Satir et al., 2007), we might expect that similar molecular features for cellular GPS function of the cilium might be present from spirotrichs to vertebrate cells.

To account for our results, when a mirror-image doublet is produced, we postulate that the orientation of the OPC on the mirror-image side of the OA is altered such that its anterior–posterior gradient remains intact, but its left–right sides are reversed. In both left and right primordia, morphogenesis of the MID OA begins similarly and ciliogenesis proceeds normally, but as maturation proceeds in the right primordium, the developing oral primordium undergoes rotation to maximize the normal association of basal bodies with the proper side of the OPC. Because the typical association places doublets 7–9 of the basal body on the left, the left–right reversal of the OPC requires 180° planar rotation of the basal body for final positioning and consequent reversal of effective stroke direction of its cilium. The oldest membranelles mature and therefore rotate first, ultimately being positioned posteriorly (caudal) in the mirror-image OA. A similar misalignment of the OPC would account for misalignment of basal bodies in respiratory epithelial cells, leading to ineffective mucociliary clearance.

When mirror-image doublets encyst, the OPC could persist in the cortex of the cell, both in its normal orientation to mark the typical OA and in its rotated state to mark the site of the mirror-image OA. When the cells excyst, the newly forming basal bodies would assemble as primordial and align themselves at the sites where the OPC persists, as before in the different orientations for the left and right oral structures. Because the OPC remains oriented in mirror image fashion at the site as excystment proceeds, the basal bodies associated with the mirror image OA will again rotate to form a new nonfunctional mouth.

Complete mirror imagery in a mirror-image doublet would require the production of basal body and therefore axonemal enantiomorphs as diagrammed in Fig. 4c. Enantiomorph forms have not been observed for any basal body or centriole. If such a form could be assembled, the prion-like informational system we have postulated would permit true mirror imaging of the membranelles to give a functional OA. However, in typical cells, some additional mechanism would then be required to ensure that mirror-image basal bodies were not produced. Otherwise, such basal bodies might be incorporated into typically oriented ciliary structures, thereby disrupting operation of the organelles, leading to selective disadvantage for the ciliate and elimination.

Detailed examination of this process at both the structural and molecular level would be necessary in order to test this hypothesis. Identification of cortical component molecules such as the OPC that are responsible for positioning and orientation of organelles, particularly of developing basal bodies, or centrioles in other cell types, will be a crucial next step toward understanding the phenomenon of cortical inheritance. In spirotrichs, this is likely to be facilitated by the ongoing genome sequencing of *Oxytricha trifallax* (Doak et al., 2003), which is closely related to *T. pustulata*.

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