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Detection of gravity-induced polarity of cytoplasmic streaming in *Chara*

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Summary. Gravity induces a polarity of cytoplasmic streaming in vertically-oriented internodal cells of characean algae. The motive force that powers cytoplasmic streaming is generated at the ectoplasmic/endoplasmic interface. The velocity of streaming, which is about 100 μm/s at this interface, decreases with distance from the interface on either side of the cell to 0 μm/s near the middle. Therefore, when discussing streaming velocity it is necessary to specify the tangential plane through the cell in which streaming is being measured. This is easily done with a moderate resolution light microscope (which has a lateral resolution of 0.6 μm and a depth of field of 1.4 μm), but is obscured when using any low resolution technique, such as low magnification light microscopy or laser Doppler spectroscopy. In addition, the effect of gravity on the polarity of cytoplasmic streaming declines with increasing physiological age of isolated cells. Using a classical mechanical analysis, we show that the effect of gravity on the polarity of cytoplasmic streaming cannot result from the effect of gravity acting directly on individual cytoplasmic particles. We suggest that gravity may best be perceived by the entire cell at the plasma membrane-extracellular matrix junction.

Keywords: *Chara*; Cytoplasmic streaming; Gravity; Vacuolar streaming.

Introduction

Plant cells exhibit a variety of patterns of cytoplasmic streaming (Kamiya 1959). In large cells, in which diffusion alone cannot transport molecules throughout the cell in a physiologically meaningful time frame, cytoplasmic streaming provides the convective movement necessary to enable biochemical reactions to proceed at their typical rates ($\approx 10^3$/s). Thus, in general, the larger the cell, the more organized and rapid is the streaming.

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Characean internodal cells are large (≈0.5 mm wide and 3 cm long) and consequently they exhibit a highly organized and rapid (100 μm/s at 298 K) rotational cytoplasmic streaming. The motive force for streaming is generated by the sliding of myosin along the actin cables that reside at the interface between the ectoplasm and endoplasm (Kamitsubo 1972; Kamiya and Kuroda 1956, 1965; Kersey et al. 1976; Nagai and Rebhun 1966; Palevitz and Hepler 1975; Shimmen and Yokota 1994; Tazawa and Shimmen 1987). The velocity of cytoplasmic streaming in characean cells is affected by many external stimuli, including light, temperature, touch, Ca^{2+} and pH (Ewart 1903, Kamiya 1959, Plieth and Hansen 1992, Tazawa and Shimmen 1987).

Ewart (1903), using high magnification optics, first noticed that the rotational cytoplasmic streaming in *Nitella* internodal cells is affected by gravity. He found that gravity induces a polarity of cytoplasmic streaming such that the downstream is 10% faster than the upstream, and the effect was greatest at the ectoplasmic/endoplasmic interface. Ewart's observations were later extended to *Chara* by Hayashi (1957) who, using ×80 total magnification, observed a gravity-induced polarity of streaming of 4.2%. However, Hayashi observed that gravity has the greatest effect at the innermost layer of the endoplasm. We (Wayne et al. 1990), using ×320 total magnification, extended the observations on the effect of gravity of *Nitellopsis*. Like Ewart, who used high magnification optics, we found that gravity induces a 10% polarity of streaming with its greatest effect near the actin

bundles. Later we also found that gravity induced a similar polarity of streaming in *Chara* using both a stopwatch technique and an automated image processing technique (Staves et al. 1992). The rhizoid cells of *Chara* are also responsive to gravity: Hejnowicz et al. (1985) observed that gravity enhances an endogenous polarity of streaming in rhizoids using a streak technique and Buchen et al. (1991) showed that the gravity-induced enhancement is diminished in rhizoids streaming in reduced gravitational fields.

A gravity-induced polarity of cytoplasmic streaming is also observed in cells of higher plants. Ewart (1903) and Bottelier (1934) observed that gravity induces a polarity of cytoplasmic streaming of 11%, 4.4%, and 29% in *Vallisneria, Elodea,* and *Avena*, respectively. Ewart (1903) initially suggested that gravity may act on each individual particle. Kamiya (1959) stated that this conclusion was unlikely, given the small density differences between particles and the high viscosity of the endoplasm. Kessler (1979) has suggested that the gravity-induced polarity of cytoplasmic streaming may be an important component of differential growth. We have used the data obtained from studying the polarity of cytoplasmic streaming in characean internodal cells as a basis for offering a novel model for gravisensing in algal, higher plant and animal cells. In this model, the most likely candidates for the gravireceptor(s) are not starch grains, but integrin-like proteins, localized at the plasma membrane-extracellular matrix junction, that sense tension at the top of the cell and compression at the bottom of the cell (Staves et al. 1992; Wayne et al. 1990, 1992).

Recently, Ackers et al. (1994) have been unable to discern an effect of gravity on the polarity of cytoplasmic streaming in characean cells, and they have questioned the existence of such an effect. They used laser Doppler spectroscopy in contrast to the usual microscopic observations using a "stopwatch technique". However, laser Doppler spectroscopy yields a spectrum of velocities and not just a single velocity and thus the spectrum must be interpreted. For example, the width of the peak depends on the heterogeneity in particle size, since large particles contribute unduly to the spectrum because the intensity of the signal is proportional to the square of the mass of the particle (Mustacich and Ware 1976; or, according to Mie, related to the sixth power of the radius, and the square of the refractive index of the particle, Drain 1986). Thus if there is heterogeneity in the size of the particles, large particles will contribute unduly to the signal. This difficulty may be dramatic in cells since the streaming particles include vesicles approximately 1 μm in diameter (which are resolvable with an objective lens with an NA greater than 0.25) as well as 20–60 μm nuclei in the endoplasm and crystals of a similar size in the vacuole.

The width and position of the peak of the laser Doppler spectrum will also depend on the depth of field of the objective lens. For example, if there is a velocity gradient in the cell and the depth of field of the objective lens is sufficiently small, laser Doppler spectroscopy will be able to distinguish the velocity gradient and the position of the peak will be determined by the plane of focus. If the depth of field is large, the peak will be broadened, reflecting the fact that particles of different velocities are being sampled. The position of the peak will not be an arithmetic average, even if the streaming particles are homogeneous, since more fast particles will be sampled in a given time than slow particles. This will result in the faster particles being over-represented in the spectrum.

The peak of the laser Doppler spectrum, therefore, does not directly indicate the average velocity of the flowing endoplasm, but must be interpreted by combining experimental data with theoretical distributions. This may be, in part, the reason that Ackers et al. (1994) concluded that cytoplasmic streaming in *Chara* is approximately half as fast and five to ten times more variable than we measure. Their interpretation that the laser Doppler spectrum yields "the velocity of cytoplasmic streaming" may contribute to their inability to resolve an effect of gravity.

The inability of Ackers et al. (1994), using laser Doppler spectroscopy, to observe an effect of gravity on cytoplasmic streaming has led them to reject our work (using the "stopwatch method"). Astonishingly, however, Ackers et al. (1994) accept Ewart's (1903) observations (using the "metronome technique") that gravity induces a 10% polarity of cytoplasmic streaming in the internodal cells of *Nitella*. Ackers et al. also purport that while it is possible to measure a gravity-induced enhancement of an endogenous polarity in *Chara* rhizoids (Hejnowicz et al. 1985), it is impossible to detect an influence of gravity on cytoplasmic streaming in internodal cells of *Chara* and *Nitellopsis* (Staves et al. 1992, Wayne et al. 1990), where there is no endogenous polarity. We are unable to comment on the logic of these arguments.

Here we present some features of cytoplasmic streaming important in evaluating the effects of gravity on streaming, and the influence of the optical system in discerning these features.

Materials and methods

Cell culture

Chara corallina Klein ex Wild., em. R. D. W. (= *Chara australis* R. Brown) was cultured in aquaria containing soil water medium as described in Wayne and Staves (1991). Under our growth conditions, $CaCO_3$ bands are absent. Moreover, the starch grains in the chloroplasts are small or absent so that the cells are translucent enough so that windows are not necessary to see small streaming particles.

Observation of cells

Freshly isolated, apical internodal cells of *Chara* (2–3 cm long) were mounted in a glass/leucite chamber containing pCa 5 buffer (Wayne et al. 1990). The chamber was placed on the stage of a horizontally oriented Olympus CH-2 microscope equipped with a ×20 (A20PL, NA = 0.4) objective lens and ×10/18L CWHK oculars.

Cytoplasmic streaming was measured with the chloroplasts in focus in order to sample the streaming rate at the site of the generation of the motive force. Streaming velocity was determined by measuring the time required for cytoplasmic particles to stream 250 μm. Twenty-five measurements were made in each direction and the polar ratio was determined for vertically oriented (gravity stimulated) cells by calculating the ratio of the velocity of the downwardly-directed stream to that of the upwardly-directed stream. The human reaction time using the "stopwatch method" is less than 0.04 s. This is approximately 1.6% of the time required for the particles to traverse 250 μm. After 25 measurements, the standard error is ca. 0.7 μm/s or about 0.7% of the streaming velocity.

In order to determine the effect of depth of field on the measurement of streaming velocity and the polarity of cytoplasmic streaming, we used lenses with differing depths of field, including Olympus ×20 (A20PL, NA = 0.4) and ×10 (A10PL, NA = 0.25) objectives, a Spencer infinity-corrected ×4 (actually ×2.4 for a 160 mm tube length; NA = 0.1) objective, and a Zeiss ×3.2 (NA = 0.07) objective lens. In order to keep the measurement times relatively constant, the distances traversed by the particles, for the different optical configurations, were 250, 200, 388, and 294 μm, respectively.

The depth of field (or axial resolution) is defined as the distance between the nearest and farthest object planes in which the objects are in acceptable focus. We are unaware of any simple derivation that helps one derive the equation most often used for determining the depth of field (Delly 1988). Because of the importance of the depth of field, we present a derivation using Abbe's criterion and simple trigonometry.

We start with Abbe's criterion for calculating the minimum distance (X, in m) between two points in which those two points can be resolved:

$$X = \lambda/(2NA) \quad (1)$$

where λ is the wavelength of the observation light (in m), λ is assumed to be 0.5 μm for white light; NA is the numerical aperture of the lens and is defined as n (sinθ) where n is the refractive index of the medium in which the objective lens is immersed and θ is the angular aperture of the objective lens (Fig. 1).

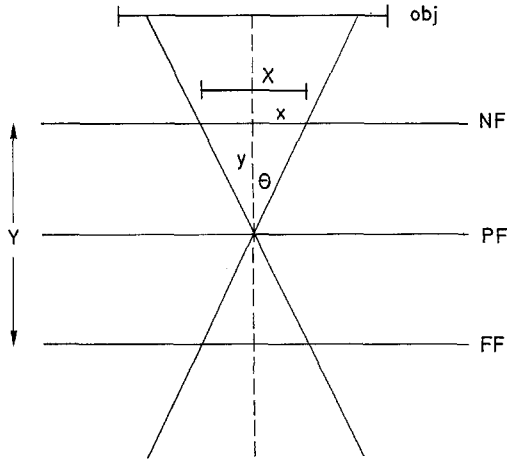

Fig. 1. Ray diagram used to determine the equation for calculating the depth of field from the numerical aperture of an objective lens. Y is the depth of field, y the distance from the plane of perfect focus to the plane of acceptable focus, X the size of the "zone of confusion" (based on Abbe's criterion), x = X/2, θ the angular aperture of the objective lens or one half of the angle that subtends the cone of light that enters the objective. *PF* Plane of perfect focus; *NF* and *FF* nearest and farthest planes of acceptable focus, respectively; *obj* objective lens

Then we make the assumption that X represents the size of an object that will be in acceptable focus at the nearest and farthest object plane. This distance is often called the "zone of confusion." The depth of field (Y) is the distance between the plane of nearest and farthest acceptable focus (Fig. 1).

Let x = X/2 and y = Y/2. Thus

$$2x = \lambda/(2NA) \text{ and } x = \lambda/(4NA). \quad (2)$$

Given the definition of tangent:

$$\tan\theta = x/y \text{ and } x = y \tan\theta. \quad (3)$$

Substituting Eq. (2) into Eq. (3) we get

$$y(4NA) = y \tan\theta. \quad (4)$$

After solving for y we get

$$y = \lambda/((4NA)(\tan\theta)). \quad (5)$$

Since depth of field (Y) is equal to 2y

$$Y = \lambda/((2NA)(\tan\theta)). \quad (6)$$

Remember that

$$\tan\theta = (\sin\theta)/(\cos\theta). \quad (7)$$

Multiply the right side of the equation by one (n/n)

$$\tan\theta = (n \sin\theta)/(n \cos\theta). \quad (8)$$

Simplify using the definition of numerical aperture

$$\tan\theta = NA/(n \cos\theta). \quad (9)$$

Remember that $\cos^2\theta + \sin^2\theta = 1$ and $\cos^2\theta = (1 - \sin^2\theta)$. Thus

$$\cos\theta = \sqrt{(1 - \sin^2\theta)}. \quad (10)$$

After substituting Eq. (10) into Eq. (9) we get

$$\tan\theta = NA/(n \sqrt{(1 - \sin^2\theta)}). \quad (11)$$

Since $n = \sqrt{n^2}$

$$\tan\theta = NA/\sqrt{[n^2(1 - \sin^2\theta)]}. \quad (12)$$

Distribute the n^2 on the right side to get

$$\tan\theta = NA/\sqrt{(n^2 - n^2\sin^2\theta)}. \quad (13)$$

Simplify, since $(n^2\sin^2\theta) = (n\sin\theta)^2 = NA^2$

$$\tan\theta = NA/\sqrt{(n^2 - NA^2)}. \quad (14)$$

Substitute Eq. (14) into Eq. (6) to get

$$Y = \lambda/[(2NA)(NA)/\sqrt{(n^2 - NA^2)}]. \quad (15)$$

Simplify

$$Y = \lambda\sqrt{(n^2 - NA^2)}/2NA^2. \quad (16)$$

Equation (16) is an equation that relates the depth of field to the numerical aperture of the objective lens. This equation is based on the validity of the Abbe criterion, the assumption that the "zone of confusion" is equal to X, and the use of an illumination where the full NA of the lens is utilized. A similar form of Eq. (16) is attained when Rayleigh's criterion for resolution ($X = 0.61\lambda/NA$) is used instead of Abbe's criterion:

$$Y = 0.61\lambda\sqrt{(n^2 - NA^2)}/NA^2. \quad (17)$$

In essence, Eqs. (16) and (17) state that the depth of field is proportional to the wavelength of light and decreases as the numerical aperture of the objective lens increases. Thus for a narrow depth of field, as is prerequisite for the observation of a localized plane of streaming, one needs an objective lens with a fairly high magnification and numerical aperture.

We developed an alternative method to measure the effect of gravity on the polarity of cytoplasmic streaming (Staves et al. 1992) using an automated image processing technique. Cells in a glass/leucite chamber were mounted on a horizontal Olympus BH-2 microscope equipped with a ×20 objective (SPLAN 20, NA = 0.46, total magnification of microscope = ×100). An image of the streaming cytoplasm was collected by a video camera and processed using the motion enhancement mode of an Image 1/AT processing and analysis system, and viewed on a video monitor. The processed images were recorded onto a video tape and digitized by a video processor (Model VP 110; Motion Analysis Corp., Santa Rosa, CA, U.S.A.). The digitized data were analyzed with a custom made computer program using the ExpertVision software to determine the velocities of the particles. In the process it eliminated any artifactural nonlinear paths, large particles (i.e., near median sections of nuclei), as well as paths with unrealistic velocities (i.e., < or > 2 × SD). The results obtained with this automated system confirmed precisely the results using the stopwatch method (Table 1).

In order to measure the thickness of the cytoplasm, cells were mounted on an Olympus BH-2 horizontal microscope equipped with a ×20 objective (SPLAN 20, NA = 0.46, total magnification of microscope = ×100). The image was collected by a video camera where it was analog enhanced and then passed through an Image 1/AT digital image processor, through a time/date generator to a video recorder and monitor (total magnification = ×1754). The recorded image was played back through the monitor and stopped every 2 s. The thickness of the cytoplasm was measured with a ruler. This was repeated until 100 measurements were made on each side of the cell.

To produce photographs, an internodal cell was observed with an Olympus BH-2 microscope equipped with a ×10 objective (A10PL, NA = 0.25) using phase optics. Images of the streaming endoplasm and vacuole were collected by a video camera using the kymograph mode of an Image 1/AT digital image processor. Photographs were taken from the monitor using TMAX 100 film and negatives were printed through a Ronchi ruling, which was used as a spatial filter.

Table 1. A comparison of streaming rates of gravity-stimulated cells obtained by eye using the stopwatch method or by an automated image processing technique

	Orientation	Direction	Streaming rate (μm/s)[a]		
			eye	automated	automated − eye
Cell 1	horizontal	right	100	101	1
	horizontal	left	102	103	1
	vertical	up	104	101	−3
	vertical	down	110	107	−3
Cell 2	horizontal	right	107	107	0
	horizontal	left	107	107	0
	vertical	up	109	116	7
	vertical	down	119	129	10
Cell 3	horizontal	right	102	104	2
	horizontal	left	103	105	2
	vertical	up	105	111	6
	vertical	down	115	120	5

[a] Paired t-tests indicate that results obtained by eye using the stopwatch method and by the automated image processing technique are not significantly different ($p = 0.71$)

Results and discussion

Cytoplasmic streaming velocity

Ackers et al. (1994) reported much lower velocities of cytoplasmic streaming (≈50 μm/s) than the velocities we (Staves et al. 1992; Wayne et al. 1990, 1992) report (≈100 μm/s). In order to determine whether the differences in velocities could be related to differences in cell maturity, we measured streaming velocities in cells sampled along the axis from apex to base, since Ackers et al. (1994) utilized cells at random whereas we typically use only the apical or subapical cell for gravity experiments. We find that the velocity of streaming at the ectoplasmic/endoplasmic interface decreases from 101.2 ± 2.1 μm/s in the apical cell to 86.5 ± 7.7 μm/s in the fifth internodal cell, indicating that this factor alone is not enough to account for the observed differences.

We also tested the effect of aging after the cells had been cut, since we typically use freshly isolated cells for gravity experiments while Ackers et al. use cells that had been isolated 3–4 days prior to the experi-

ment. The streaming rate of cells remains stable at about 106 μm/s for 5 days after cutting, and then slowly declines to about 87 μm/s after 7 days. Thus cell aging is not responsible for the disparate velocities reported by us and Ackers et al. (1994).

Using laser Doppler spectroscopy, Ackers et al. (1994) concluded that all particles in the endoplasm travel at the same velocity and that velocity corresponds to the peak in the Fourier spectrum (i.e., that the endoplasm moves as a plug). The original interpretation that the endoplasm moves by plug flow is based upon observations made with a shadowgraph that had a total magnification of ×50 (Kamiya and Kuroda 1956). However, particles travelling at different velocities are readily apparent when young, relatively transparent cells are observed with magnifications of ×100 or greater. Using high magnification (×100 objectives), Kamitsubo (1972) felt that the velocity of the protoplasm was highest at the ectoplasmic/endoplasmic interface in *Nitella*. Such a velocity gradient has been observed by Mustacich and Ware (1977) in *Nitella* and by us (Wayne et al. 1990) in *Nitellopsis*. Other experiments by Mustacich and Ware (1976) using laser Doppler spectroscopy are consistent with the presence of a velocity gradient in the endoplasm. Since the full width at half height (10%) of the laser Doppler peak is too wide to represent plug flow and too narrow to be Poiseuille flow, Mustacich and Ware (1976) concluded that the endoplasm does not move as a plug but that the endoplasmic flow is intermediate between plug flow and parabolic flow. This is consistent with a velocity gradient across the endoplasm.

The Fourier spectrum obtained by laser Doppler spectroscopy also shows that some particles move at velocities slower and faster than those represented by the peak. It is important to ask what organelles are represented by the various parts of the spectrum. Mustacich and Ware (1974) observed that the velocities represented by the main peak are correlated with the velocities of very large particles seen in microscopes, consistent with the fact that the magnitude of scattered light is proportional to the square of the mass of the particle (Mustacich and Ware 1976). The large particles observed at low magnification are probably nuclei in the endoplasm and crystals in the vacuole. The identities of the particles that give rise to the smallest and largest Doppler shifts have not been determined. However, the spectral density of these shifts, as well as that represented by the peak, drop to zero upon the stoppage of streaming (Mustacich and Ware 1974). Thus the Fourier spectrum results from an assortment of velocities.

The Fourier spectrum presented by Ackers et al. (1994), like those presented by Mustacich and Ware (1974), clearly shows a multiparted spectrum, with a main peak as well as a shoulder that may represent slower particles and a tail that may represent faster particles. The full width at half height of the spectrum presented by Ackers et al. is similar to that presented by Mustacich and Ware (1974), indicating that there is a 10–20% variation in the particle velocities and that the endoplasm does not move by plug flow. Our use of an objective lens with a moderate numerical aperture (and ×200 total magnification) allows us to limit our observations to the site of the fastest particles (along the ectoplasmic/endoplasmic interface). Ackers et al. (using an objective lens with a low numerical aperture and a total magnification of ×50) are most likely reporting the velocity of the largest particles throughout the endoplasm (possibly including the vacuole).

Ackers et al. (1994) documented a large variation (10–20%) in the streaming rates of horizontal cells. By contrast, we observe a relatively constant streaming rate in horizontal cells using the "stopwatch technique". We find that the difference in velocity of the two opposing streams in a horizontal cell never exceeds 2%. Using our method, then, the variation in opposing streaming velocities is small enough to be able to discern a difference in polarity induced by gravity. Mustacich and Ware (1974), also using laser Doppler spectroscopy, never saw a large velocity variation in horizontal "fresh and healthy cells", in contrast to the results reported by Ackers et al. (1994). Ackers et al. conclude that in terms of statistics, the large variability that they see in horizontal cells should obscure any effect of gravity on cytoplasmic streaming.

The effect of gravity on the polarity of cytoplasmic streaming

Gravity induces a polarity of cytoplasmic streaming in *Chara* of 1.087 ± 0.013 as measured with a light microscope at the ectoplasmic/endoplasmic interface using the "stopwatch method". A polarity of 1.083 ± 0.014 is obtained using an automated image processing technique (Staves et al. 1992) (Table 2). The close similarities of streaming rates and polar ratios induced by gravity measured by the two tech-

Table 2. A comparison of polar ratios of gravity-stimulated cells obtained by eye using the stopwatch method or by an automated image processing technique

	Orientation	Polar ratio[a]		
		eye	automated	automated – eye
Cell 1	horizontal	1.02	1.02	0
	vertical	1.06	1.06	0
Cell 2	horizontal	1.00	1.00	0
	vertical	1.10	1.11	0.01
Cell 3	horizontal	1.01	1.01	0
	vertical	1.10	1.08	–0.02

[a] Paired t-tests indicate that the results obtained by eye using the stopwatch method and by the automated image processing technique are not significantly different ($p = 0.95$)

niques show an excellent congruence between the "stopwatch method" and the automated one.

We tested the effect of aging on the ability of isolated internodal cells to sense gravity and found that the polarity of cytoplasmic streaming decreases as the cells age (Fig. 2). Since Ackers et al. (1994) used cells that had been cut for 3–4 days before use, their inability to observe an effect of gravity may have been due, in part, to the physiological state of the material.

Since there is no accumulation of cytoplasm at the bottom of vertical cells, the slower upwardly-directed stream must be thicker than the faster downwardly-directed stream. This prediction is borne out by experiment. In horizontal cells, the endoplasm travel-

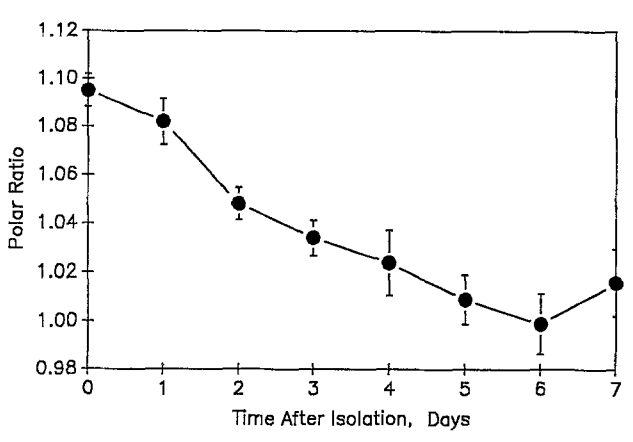

Fig. 2. The effect of age of isolated *Chara* internodal cells on the gravity-induced polarity of cytoplasmic streaming. In addition to a decrease in the polar ratio, the average streaming velocity decreases from 102 ± 0.9 μm/s in freshly cut cells to 87 μm/s on day 7. Mean values ± SE are presented (n = 8–10)

Table 3. The thickness of the cytoplasm[a] measured from the cell wall to the vacuolar membrane

Orientation	Direction	Thickness (μm)	n	Level of significance[b]
Horizontal	left	14.96	6	0.49
	right	14.95	6	
Vertical	up	17.04	6	0.007
	down	14.61	6	

[a] The thickness of the cytoplasm includes both the ectoplasm and the endoplasm. The ectoplasm alone is approximately 3 μm thick

[b] Using a one-tailed Student's t-test

ling in either direction has an average thickness of 11.95–11.96 μm. However, in vertically-oriented internodal cells, the slower upstream increases in its mean thickness to 14.04 μm while the downstream decreases to 11.61 μm (Table 3). The difference in the average thickness of the two streams ensures that the endoplasm flows continuously with neither a breakage nor an accumulation.

We always observe a decrease in the velocity of streaming as we focus at planes more distant from the ectoplasmic/endoplasmic interface in *Chara* internodal cells (Fig. 3 A). A similar velocity gradient has been observed in vertical *Nitellopsis* cells (Wayne et al. 1990). Given the average velocity gradient of 14.58 μm/s and the average thickness of the endoplasm of 12.39 ± 0.56 μm, we calculate the average rate of shear (velocity/thickness) in the endoplasm of *Chara* is 0.85/s.

The velocity gradient continues through the vacuolar membrane into the vacuole. The contents of the vacuole move as a consequence of the undulations in the vacuolar membrane that result from the protrusion of hundreds of nuclei, 20–60 μm in diameter, into the vacuole (Figs. 4 and 5). Near the middle of the vacuole, the streaming velocity approaches zero due to the distance of the particles from the site of motive force generation in one direction and their increasing proximity to the site of generation of the oppositely directed motive force. It follows from the continuity laws of fluid dynamics (Duncan 1990) that a technique that measures particles throughout the thickness of the upwardly- and downwardly-directed streams will yield identical average velocities and would fail to measure a polar ratio.

As with the velocity, the polar ratio is greater at the

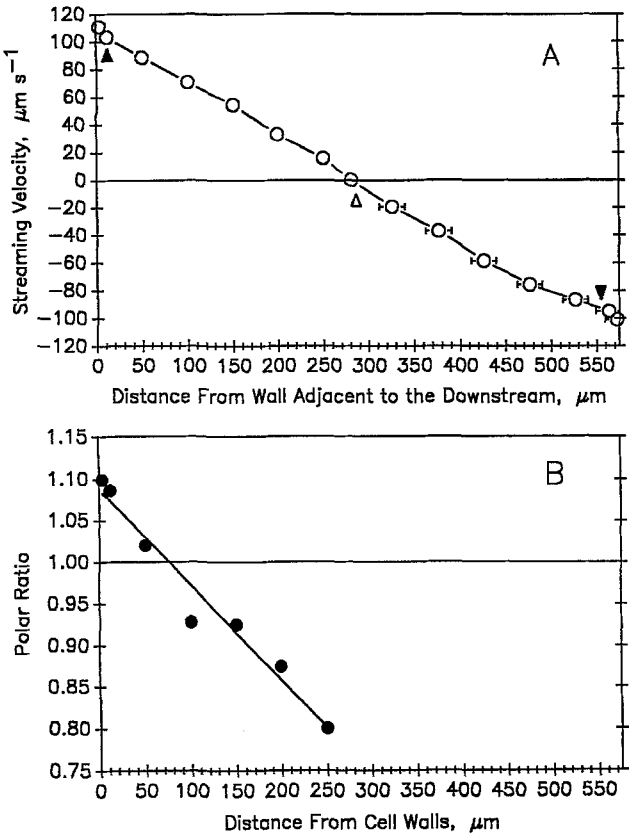

Fig. 3. Streaming velocities and polarities in a vertical cell at various distances from the cell wall. **A** Profile of the velocity of cytoplasmic streaming in vertically-oriented internodal cells of *Chara*. △ Middle of the cell; ▼, ▲ position of the vacuolar membrane. **B** The polar ratios at various distances from the cell wall are also presented. The polar ratios are greater than one when streaming is observed near the ectoplasmic/endoplasmic interface and decrease as the focal plane moves to more interior portions of the cell. Eventually the polar ratio decreases to less than one as a consequence of the greater thickness of the upwardly-directed stream compared to the downwardly-directed stream at a given distance away from the ectoplasmic/endoplasmic interface. The correlation coefficient and level of significance of the linear regression are 0.962 and 0.0001, respectively (n = 4)

ectoplasmic/endoplasmic interface. The gravity-induced polar ratio can also be observed within the vacuole near the vacuolar membrane, but as one approaches the middle of the cell the polar ratio declines and eventually becomes less than one (Fig. 3 B).

Since there is a velocity gradient across the cell, the depth of field of the objective lens will have a major impact on the observed velocity. In Fig. 6, the velocity and polarity observed with four different objective lenses are compared. The apparent velocity decreases as the depth of field of the objective lens increases. As the depth of field increases, particles in deeper planes of the cell become in focus, and more slower particles are observed. Also since the lateral resolution ($\lambda/(2NA)$) is inversely correlated with the depth of field (axial resolution), as the depth of field increases the ability to resolve small particles decreases. This also yields an apparent decrease in the streaming velocity since the large particles (e.g., the crystals in the vacuole and the nuclei located in the endoplasmic bulges that protrude into the vacuole) tend to be part of the slower moving regions of the cell.

We also find that the ability to resolve an effect of gravity on the polarity of cytoplasmic streaming depends on the depth of field of the objective lens (Fig. 6). The polar ratio decreases from 1.097 to 1.030 as the depth of field increases from 1.43 (×200 total magnification, and lateral resolution of 0.6 μm) to 24.87 μm (×40 total magnification, and lateral resolution of 2.5 μm). Thus, the maximal polarity of streaming is detectable only when the optical system can discern specifically the particles flowing near the ectoplasmic/endoplasmic interface.

The ability of the Doppler microscope to distinguish the velocity of individual particles also depends on the lateral and axial resolution of the objective. We estimate the depth of field of the objective used by Ackers et al. (1994) was 13.91 μm (since the numerical aperture of the ×5 objective was 0.15 and the wavelength of light was 0.633 μm). The lateral resolution of this objective was 2 μm. From the data presented in Fig. 6, we estimate that they will observe 40% of the maximal polarity given this optical system.

We find that isolated internodal cells of *Chara* have another response to gravity that does not depend on optical measurements of velocity. The polarity of Sr^{2+} flux into the ends of isolated internodal cells is dependent on the position of the cell with respect to gravity. In horizontal cells the flux into both ends is 23.7 ± 7.6 nmol/m^2/s. In vertical cells, the flux into the top increases to 64.3 ± 2.0 nmol/m^2/s, while the flux into the bottom remains almost unchanged (18.9 ± 2.7 nmol/m^2/s). Thus the polarity of flux is approximately three times greater in vertical cells compared with horizontal cells (Fig. 7). Interestingly, the gravity-induced Sr^{2+} flux is affected by the same factors that affect the gravity-induced polarity of cytoplasmic streaming (aging, external Ca^{2+} concentrations, Ca^{2+} channel blockers, temperature, osmotic pressure, peptides that interfere with integrin binding, etc.). These data will be presented in detail elsewhere.

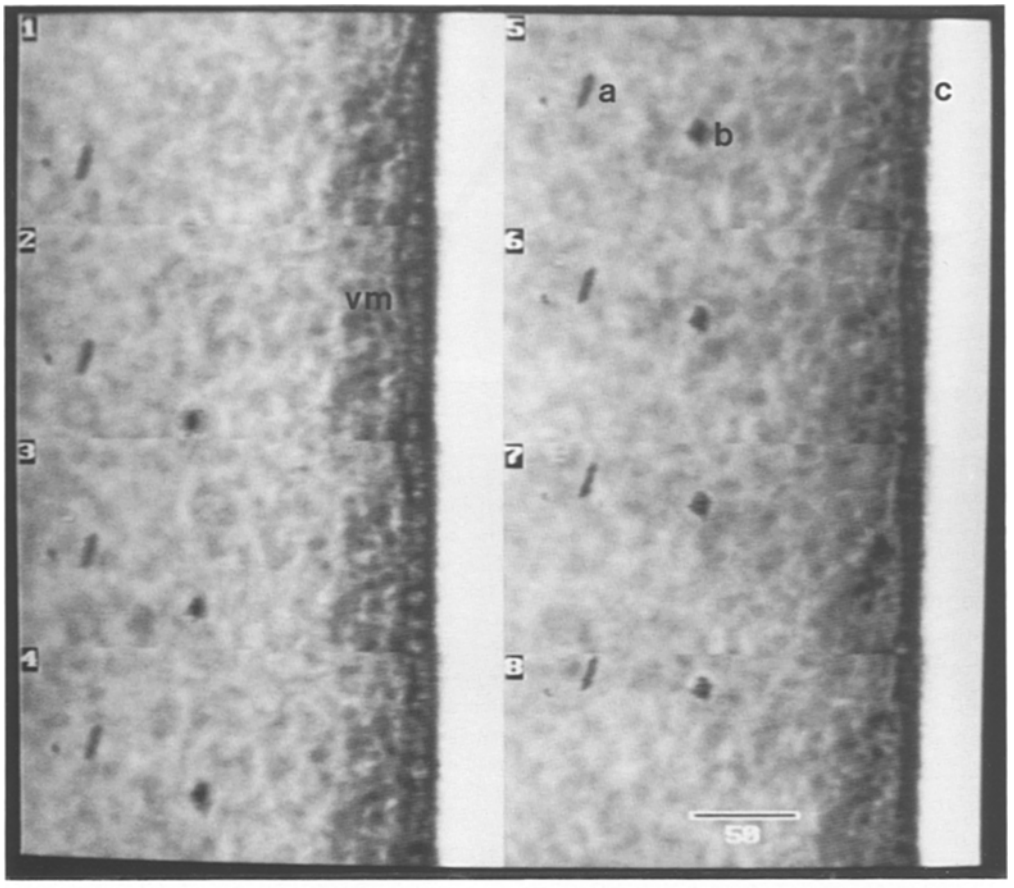

Fig. 4. Light micrographs of the cytoplasm of a horizontal *Chara* internodal cell showing large particles moving in the vacuole, the undulations of the vacuolar membrane (*vm*) and particles moving in the endoplasm. Pictures (*1–8*) were taken sequentially at a rate of 5/s using a kymograph. Particles *a* and *b* are in the vacuole whereas particle *c* is in the endoplasm. Particles *a*, *b*, and *c* are travelling at velocities of 50, 80, and 104 μm/s, respectively. The particles are 158, 105, and 8.3 μm from the wall, respectively

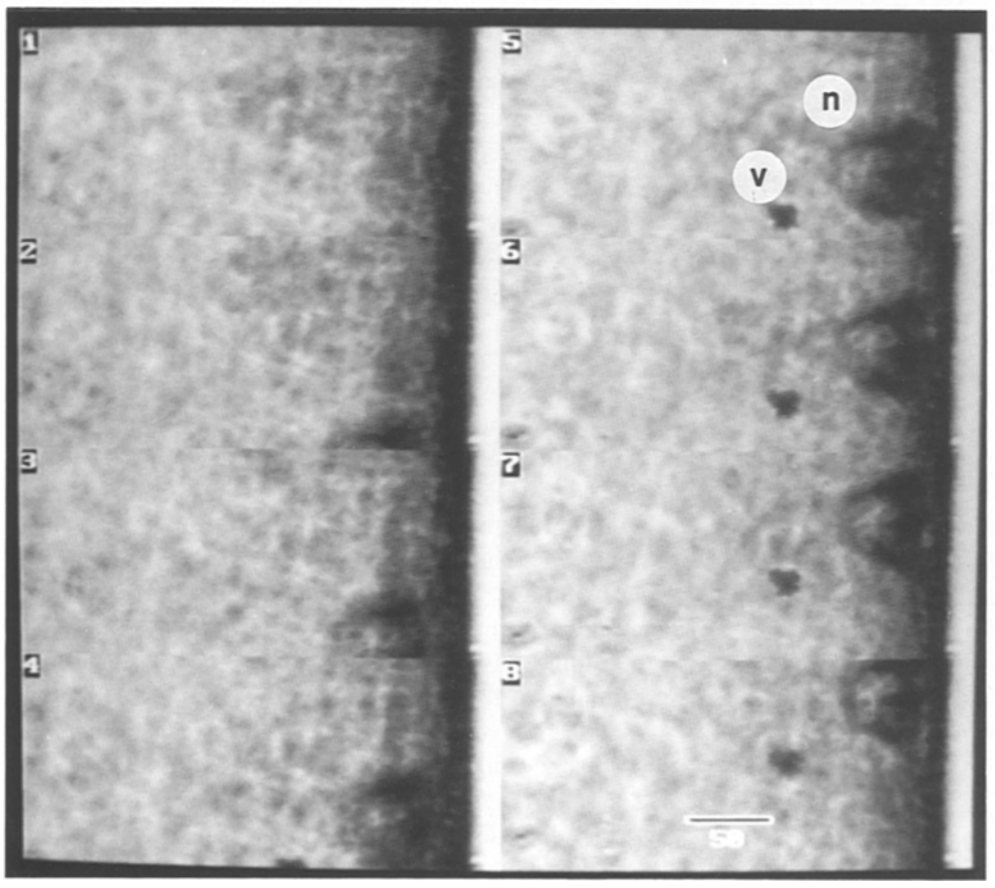

Fig. 5. Light micrographs of the cytoplasm of a horizontal *Chara* internodal cell showing the distension of the vacuolar membrane by a passing nucleus. Pictures (*1–8*) were taken sequentially at a rate of 5/s using a kymograph. The nucleus (*n*) and vacuolar particle (*v*) are travelling at velocities of 99 and 76 μm/s, respectively. The innermost portion of the nucleus and the vacuolar particle are 51 and 76 μm from the wall, respectively

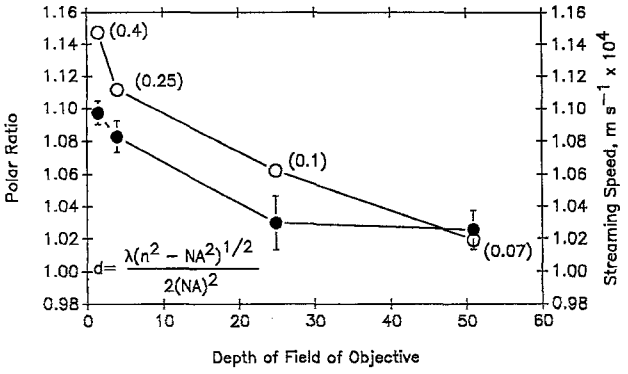

Fig. 6. The effect of the depth of field of the objective on the apparent velocity of cytoplasmic streaming (○) and the polar ratio (●). The numerical aperture of the objectives is given in parentheses. d is the depth of field, λ the wavelength of light, n the refractive index of the medium, and NA the numerical aperture of the objectives

Ackers et al. (1994) imply throughout their paper that the laws of physics support the interpretation that any physiologically meaningful effects of gravity would be undetectable and the small effect of gravity that is observed may reflect a purely physical effect on each individual particle. However, they are not explicit as to which laws they are using to make their argument. Although we are aware that not all of the known laws of physics may be applicable to biological systems (Wayne 1994), we have applied the laws of classical mechanics (Newton's Second Law and Stokes's Law) to the problem.

Ackers et al. (1994) stated that the ability to detect a gravity-induced polarity in cytoplasmic streaming was impossible given the observation that it took 148–178 **g** to stop the centripetally-directed streaming of the bulk endoplasm (Wayne et al. 1990). Based on the assumption that the effect of gravity on streaming depends on the "ratio" of the acceleration due to gravity to the centrifugal acceleration needed to stop the centripetal stream (i.e., the balance acceleration), they concluded that any effect of gravity (1 **g**) on the velocity of streaming would be undiscernible. However, we had previously demonstrated that the effect of centrifugal acceleration on streaming velocity was nonlinear (Wayne et al. 1990). We reported that low accelerations (0–30 **g**, which cause minimal accumulation of endoplasm at the centrifugal end after short times), caused a decrease in the centripetal streaming velocity, an increase in the centrifugal streaming velocity, and consequently a polarity in cytoplasmic streaming. At higher accelerations, the effect of relative centrifugal acceleration on velocity became nonlinear because the streaming endoplasm became thinner as it accumulated at the centrifugal end of the cell. Thus the force of the centrifugal acceleration became smaller since the shearing force (per unit area; F/A) due to the relative centrifugal acceleration (α) depends on the thickness of the endoplasm according to the following equation:

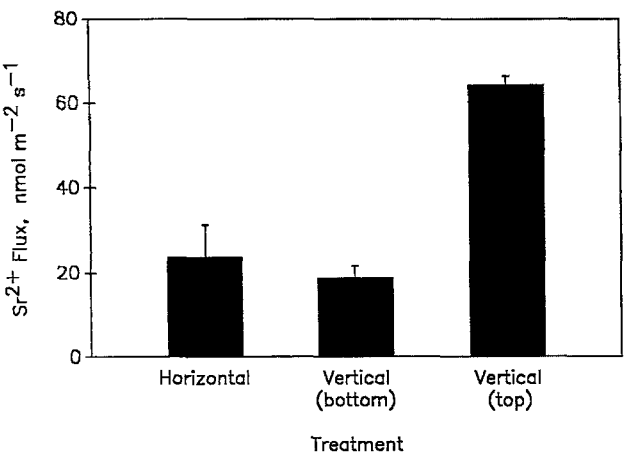

Fig. 7. The effect of gravity on the influx of Sr^{2+} into the ends of isolated internodal cells of *Chara*. Sr^{2+} influx into the ends of horizontal and the tops and bottoms of vertical cells was determined by incubating the cell ends in 100 μM Sr^{2+} for 20 min. After incubation, the cytoplasm was extruded and the [Sr^{2+}] measured with an ignition-coupled argon plasma spectrophotometer

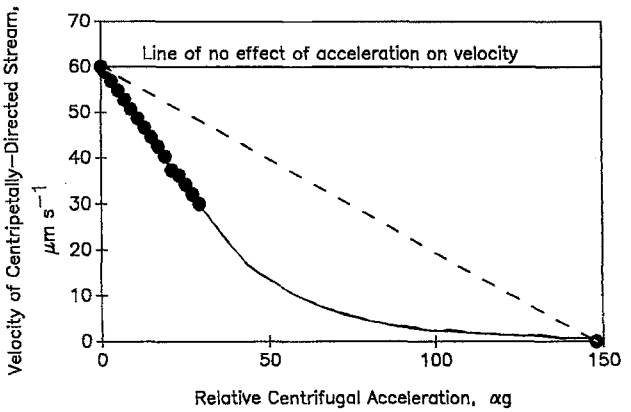

Fig. 8. Two alternative ways of interpreting the data presented by Wayne et al. (1990) on the effect of centrifugal acceleration on the velocity of the centripetally-directed stream of *Nitellopsis* at 20–21 °C. ● Nonlinear effect of centrifugal acceleration on streaming velocity. The nonlinear relation comes from the fact that the thickness of the endoplasm decreases with time at high accelerations. The dashed line represents the linear interpolation made by Ackers et al. (1994) from the balance acceleration (148–178 **g**), without taking into consideration the data represented by the other solid circles. The solid horizontal line represents the hypothetical case where the velocity of streaming does not change when the cell is subjected to centrifugal accelerations (including 1 **g**). Interestingly, extrapolation of the linear portion of our data to the ordinate may yield a better estimate of the shearing force that powers cytoplasmic streaming (0.07 N/m at 20–21 °C, using Eq. (18)), since the thickness will be minimally changed at these low accelerations

$$F = (\alpha)\,(\mathbf{g})\,(\rho_{en} - \rho_{cs})\,(A)\,(t) \quad (18)$$

where $(\rho_{en} - \rho_{cs})$ is the difference between the density of the endoplasm and the cell sap, and t is the thickness of the cytoplasm. This equation is a restatement of Newton's Second Law ($F = ma$) where $(\alpha\mathbf{g}) = a$ and $(\rho_{en} - \rho_{cs})\,(At) = m$. Thus as the endoplasm accumulates at the centrifugal end of the cell, the relative centrifugal acceleration needed to stop streaming (i.e., balance acceleration) is overestimated. Therefore until the change in thickness of the endoplasm (t) is taken into consideration, the balance acceleration must be considered to be only an estimate of the shearing force that powers cytoplasmic streaming. Due to the uncertainty of the thickness of the endoplasm under centrifugal accelerations, the linear part of the velocity-relative centrifugal acceleration curve must be used to compare centrifugal and gravitational accelerations (as was done by Wayne et al. 1990). Indeed Kamiya and Kuroda (1958) mention that the thickness of the endoplasm is the most difficult parameter to measure during centrifugation. Thus linear interpolation from nonlinear data as was done by Ackers et al. (1994) is not warranted, especially when the data relevant to the small acceleration in question (1 **g**) was available (Fig. 8). Significantly, using the data obtained from low accelerations, Wayne et al. (1990) found that the effect of centrifugal acceleration was equivalent to that of gravitational acceleration.

Ackers et al. (1994) also state that the small effect of gravity that is observed may have a purely physical (i.e., non-physiological) effect on each individual particle. Given the density of the endoplasm (ρ_{en}) of 1014 kg/m^3 (Wayne and Staves 1991), the viscosity (η) of the endoplasm of 1 Pa · s (Kamitsubo et al. 1988), and an abundant, dense, spherical particle (e.g., mitochondrion) with a density (ρ_m) of 1180 kg/m^3 and a radius (r) of 0.5×10^{-6} m, the force (F) exerted on the mitochondrion due to gravity would be -8.5×10^{-16} N, according to the following equation:

$$F = (4/3)\,(\pi r^3)\,(\rho_{en} - \rho_m)\mathbf{g} \quad (19)$$

which is a statement of Newton's Second Law. The force will cause the particle to move against the friction of the endoplasm with a velocity (v) of 9×10^{-11} m/s, according to Stoke's Law:

$$v = (F)\,(6\pi r\eta)^{-1}. \quad (20)$$

Evidently gravity would increase or decrease the velocity of the particle relative to the surrounding endoplasm, which flows with a velocity of 10^{-4} m/s, by less than 0.0001%. This would yield a polar ratio of 1.000002. Even if the microviscosity of the endoplasm were lower as suggested by Ackers et al. (1994) and as low as that of water (0.001 Pa s; the minimum for an aqueous environment) as direct effect of gravity on an individual particle would change the velocity of the particle by 9×10^{-8} m/s (0.09%). This would yield a polar ratio of only 1.002. Thus the direct effect of gravity on the individual particles cannot explain even the small effect of gravity on the polarity of cytoplasmic streaming observed by Ackers et al. (1994), who report average gravity-induced polarities of 2–3%. Moreover, a direct effect of gravity on the individual particles could not explain the reversal of the polarity of cytoplasmic streaming we reported by varying the Ca^{2+} concentration, and by varying the density of the external medium (Wayne et al. 1990).

Given that the continuity law of fluid dynamics holds, as it does in cells subjected to an acceleration of 1 **g** (where there is no accumulation of endoplasm, in contrast to cells subjected to 100 **g** in a centrifuge microscope), the only explanation we can envision for the effect of gravity on the polarity of cytoplasmic streaming is that which has been presented previously (Staves et al. 1992; Wayne et al. 1990, 1992). That is, gravity acts on the entire protoplast, which consequently "settles" within the extracellular matrix, resulting in tensile and compressive stresses at the top and bottom of the cell, respectively. These stresses are presumed to act upon integrin-like molecules at the plasma membrane-extracellular matrix junctions at the ends of the cell. The integrin-like molecules differentially activate Ca^{2+} channels on the top and bottom of the cell, resulting in a differential Ca^{2+} influx, which in turn may induce the polarity of cytoplasmic streaming.

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