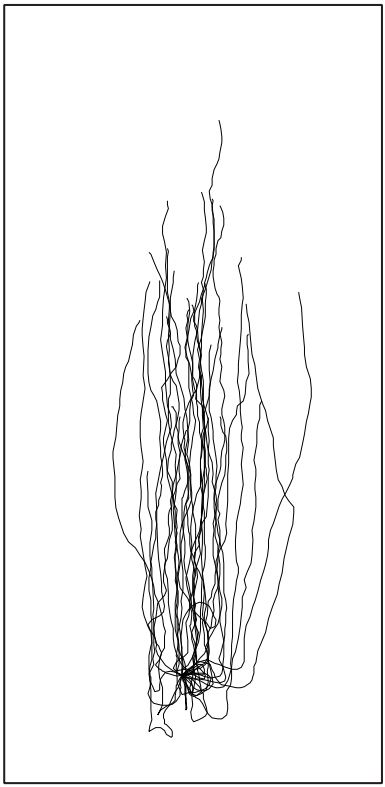

Phototaxis of *Dictyostelium discoideum* Slugs

Dissertation zur Erlangung des Grades eines
Doktors der Naturwissenschaften
-Dr. rer. nat –

der Fakultät für Biologie der
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München, Juni 2000

Vorgelegt von

Kota Miura

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Ehrenwörtlich Versicherung:

Hiermit erkläre ich, diese Arbeit selbständig verfaßt und keine als die angegebenen Hilfsmittel verwendet zu haben. Diese oder eine gleichartige Dissertation wurde nicht an einer anderen Hochschule eingereicht oder endgültig nicht bestanden.

München, den 18. 9. 2001

Kota Miura

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1 Summary

During the slug stage of cellular slime mold *Dictyostelium discoideum*, up to 10^5 cells coordinate their movement and migrate as a single organism. Slugs have a cylindrical shape with tip and tail; their morphological polarity corresponds to the polarity of migration. A large body of results suggest that cyclic AMP-mediated cell-cell signaling is the mechanism coordinating multicellular movement. Waves of cyclic AMP generated at the anterior tip propagate towards the tail and induce the chemotactic movement of cells toward the tip. Slugs exhibit highly sensitive environmental reactions: phototaxis, chemotaxis and thermotaxis. Although many studies have investigated how *Dictyostelium* slugs move toward a light source, the mechanism of phototaxis is still unclear. It has been known that slugs turn towards the light at the anterior end. In addition, previous research identified mutations and drug treatments that interfere with phototaxis but the strategy for analyzing phototaxis has been limited to low resolution both temporarily and spatially.

In this thesis methods have been developed to analyze phototactic behavior on two different scales, the slug level and cellular level. The analyses revealed dynamic features of slug behavior during phototaxis which have not been previously described. Following light irradiation slugs moved with approximately 50% higher speed; they showed prominent serpentine movement of their tip as if they were scanning and correcting migration direction; they elongated and decreased the diameter of their body; and their tip remained lifted off the substrate for long periods. The analysis of cell movement during phototactic turning showed that the cell movement pattern was unlike any predicted from earlier hypotheses. Some cells in the anterior zone moved away from the light source across the slug, thus increasing the volume on the “dark” side (“asymmetric cell accumulation”) and bending the anterior zone like a lever-arm toward the light source. Furthermore, it

was discovered that light irradiation enhances secretion of cyclic AMP from the slug and that light interferes with cyclic AMP cell-cell signaling during other multicellular stages as well. A model for phototaxis has been proposed based on these results. Laterally irradiated light is focused on the distal side of the slug by a lens effect and locally induces cyclic AMP release. Some cells accumulate chemotactically on the side away from the light source and cause a bending of the anterior zone towards the light source. Since cell movement within the slug is organized by cyclic AMP waves, light induced cyclic AMP release interferes with the endogenous signaling pattern. The consequence is an overall change in the shape and the behavior of slug. The mechanism by which light induces the release of cyclic AMP from slug cells may involve a histidine kinase phosphorelay pathway, since such a pathway is known to be functional in *Dictyostelium* and is used for environmental responses in many other organisms.

2 Introduction

A river and the drops *in this river. The position of every drop, its relation to the others; its connection with the others; the direction of its movement; its speed; the line of the movement – straight, curved, circular, etc – upwards, downwards. The sum of the movement.*

V. I. Lenin, Philosophical Notebooks

2.1 Phototaxis

Organisms sense and respond to environmental stimuli. One well known example is phototaxis; a directed movement towards a light source. The type of response is diverse, its mechanism ranging from simple to complex. In simple organisms such as bacteria, a gradient of brightness is interpreted by the movement of the individual across the space. Bacteria compare the light intensity temporally and phototaxis sets in by a change in the frequency of random turning (Hoff et al., 1994). Some unicellular protista substitute such scanning behavior by the rotation of their body. For example in *Euglena*, flagellar beating causes both swimming and the rotation of the body around the axis of swimming direction. The photosensor is located at the central axis of the body while the eye spot (so called but it is not a photoreceptor) shades the incoming light periodically because it rotates with the body. Cyclic dark-light stimulation causes the random turning. When the long-axis of body is aligned with the direction of light, the eye spot does not shade the light and the organism swims continuously. *Euglena* interprets the direction of light source as the presence of dark-light cycle that switches on and off the random movement, thus increasing the probability of swimming towards the light source (Häder, 1991). Phototaxis becomes more sophisticated in case of a multicellular protista, *Volvox*. This colonial alga

consists of several thousand cells imbedded in a matrix at the surface of the spherical colony. The cells are fixed in position but the beating of flagella rotates the colony around the axis of swimming. Unilateral light causes the cells to experience a dark-light cycle due to the rotation of colony. The alternating light stimulus deactivates and activates the flagellar beating. Since beating becomes faster in the darker hemisphere, the colony gradually turns towards the light source (Hegemann, 1997). Although cells do not communicate with each other, the colony shows a coordinated behavior because of its geometry and rotation.

These mechanisms are based on a series of functional steps to interpret the environmental information; dark-light stimulation of photoreceptor and effectors (flagellar beating) to respond. In simple systems as described above, these steps are mutually overlapping. That is to say, bacterial motion, flagella beating of *Euglena* and *Volvox* rotation have dual function of both processor and effector. In higher animals, these functional steps become discrete with specific organs such as eyes, neurons and muscles, each with higher complexity in their mechanisms. An increased complexity and the separation of functions might enable more versatile and more complex responses such as ‘recognition’.

In the multicellular *D. discoideum* slug, cells organize themselves by cell-cell communication to coordinate their movement and are able to sense and react to external signals like light and temperature. The principal aim of this thesis was to study how this multicellular complex of “social amoebae” cooperatively responds to light as a single organism.

2.2 Life cycle of *Dictyostelium discoideum*

Cellular slime moulds are unique organisms positioned between uni- and multi-cellular life in the evolutionary tree. The amoebae of the cellular slime

mould *Dictyostelium discoideum* normally live as single cells in forest litter and feed on bacteria. They multiply by binary fission. Starvation induces a developmental program in which thousands of amoebae aggregate chemotactically to form a multicellular mass (Bonner, 1947; Runyon, 1942; **Fig. 1a**). During the early stage of aggregation cells are separated from each

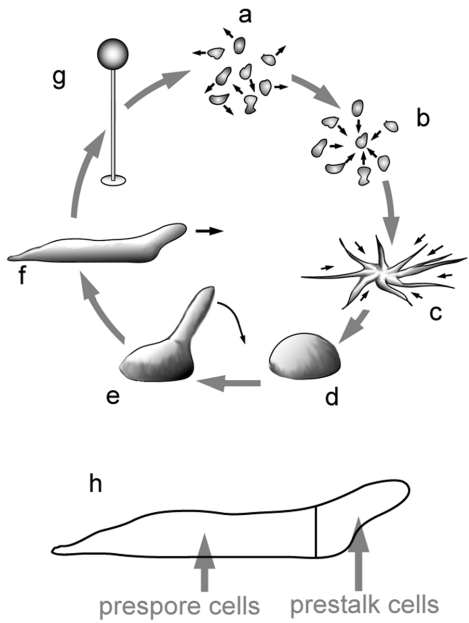

Figure 1. Life cycle of *Dictyostelium discoideum*.

Black arrows represent movement. (a) Cells in vegetative stage. (b) Early aggregation stage. (c) Late aggregation stage, or streaming. (d) Mound stage. (e) First finger stage. (f) Slug stage. (g) Fruiting body. (h) Localization of two cell types in a slug.

other. In the later stage known as streaming, cells form end-to-end cell-cell contacts and show a concerted directed movement towards the aggregation center. Aggregation results in the formation of a mound, a complex of up to 10^5 cells, followed by the upward extension of the aggregate to form a standing finger-like cell mass that then topples over and starts migration. This multicellular complex, the so called pseudoplasmodium or slug, migrates and behaves as a single organism. The slug finally culminates into a fruiting body consisting of a stalk and a mass of spores.

The slug enables cellular slime moulds to find an optimal location for fruiting body construction. *D. discoideum* slugs are highly sensitive to light, pH and even slight differences in temperature (Bonner, 1994). These abilities allow the slug to migrate towards the surface of the litter stratum and thus to have a better chance for spore dispersal after the formation of fruiting body (Loomis, 1982). Spores are dispersed by rain and small animals and under suitable conditions germinate to release amoebae and the whole cycle starts over again.

2.3 Mechanism of slug movement

2.3.1 Basic features of slug migration - shape and behavior -

Slugs have a roughly cylindrical morphology and are between 0.5 and 2.0 mm long and about 0.1 mm diameter (Raper, 1940). Their migration is driven by the coordinated movement of thousands of cells. Average migration speed ranges from 0.3 to 2.0 mm per hour, a comparable range to single cell movement. Slugs can migrate continuously for up to 20 days (Bray, 1992). The speed increases with the size of the slug (Bonner, 1995; Inouye and Takeuchi, 1979; Smith et al., 1982), decreases with slug age and at high and low temperature (Poff and Skokut, 1977). When a slug is migrating

in the absence of external stimuli, it tends to migrate straight in one direction. Turning occurs spontaneously probably due to internal physiological noise (Fisher et al., 1983). During migration slugs show a periodic “serpentine arching movement”, during which the slug tip periodically lands on and lifts off the substrate with approximately 10 minutes period (Breen et al., 1987; Dormann et al., 1996; Inouye and Takeuchi, 1979). The tip is lifted off the agar most of the time and when the slug tip lands on the agar substrate, the tip decreases its speed (Inouye and Takeuchi, 1979).

The surface of slug is covered with a layer of extracellular matrix (slime sheath) that is continuously synthesized at the front of the slug. The slug cells migrate within the slime sheath which is stationary with respect to the substrate and is left behind the advancing slug as a collapsed tube or “trail” (Bonner, 1967). Several experiments suggest that the mechanical rigidity of the slime sheath is important for normal slug migration (Wilkins and Williams, 1995). Mutant slugs with defects in slime sheath biosynthesis exhibit a slower migration or loose their integrity soon after slug formation (Breen et al., 1987; Dimond and Loomis, 1975).

Slugs have a distinct polarity with a tip at the anterior end which guides the movement of the remaining cells (Raper, 1940). This was best exemplified by tip transplantation experiments. The transplantation of a dissected tip to the prespore zone of a slug results in the splitting of the slug since cells anterior to the implant follow the host tip and cells posterior to the implant follow the transplanted tip (Rubin and Robertson, 1975). Transition from the slug to the fruiting body (culmination) is triggered by overhead light, low humidity, high temperature and low or high pH (Newell et al., 1969; Raper, 1940). Chemical substances such as cyclic adenosine 3’5’-monophosphate (cAMP), slug turning factor (STF) and gaseous ammonia (NH_3) delay the onset of culmination (Fisher et al., 1984; Fisher and Williams, 1981; Schindler and Sussman, 1977; Sussman and Schindler, 1978).

Slug consists of two major cell types; ~20% are prestalk cells localized within the anterior zone; some are scattered in the posterior zone and are called anterior-like cells; ~80% are prespore cells localized in the posterior zone (**Fig. 1**). In the mature fruiting body, prestalk cells become stalk cells and prespore cells develop into spore cells. 1~2% cells located at the rear end of the slug form the basal disc of the fruiting body. Prestalk cells are further categorized into several sub-types by differences in the expression of the extracellular matrix proteins and each of these sub-types has a specific localization within the slug anterior zone (Williams, 1997). When a slug is microsurgically separated into parts, the fragment from the anterior zone has approximately three times higher motive force per volume and moves at a higher speed than the posterior fragment, indicating that prestalk cells are innately more active than prespore cells (Inouye and Takeuchi, 1979; Inouye and Takeuchi, 1980). The difference in activity of these two zones is further supported by the fact that the prestalk cells move at 40% higher speed *in vivo* (Siegert and Weijer, 1992) and that the dissociated prestalk A cells, a major prestalk sub-type, move faster towards the artificial cAMP source than prespore cells (Early et al., 1995).

2.3.2 Cell-cell signaling coordinates cell movement

cAMP is the primary chemoattractant for the cells during early aggregation. cAMP is emitted from the aggregation center in a pulsatile manner and surrounding cells detect it by highly specific cAMP receptors (Parent and Devreotes, 1996). Since each cell responds to cAMP by moving towards the source of cAMP, by emitting a pulse of cAMP itself and by a period of adaptation, the cAMP signal is relayed outward from the aggregation center as a wave (Dinauer et al., 1980b; Konijn et al., 1967; Roos et al., 1975; Shaffer, 1975). Phosphodiesterase secreted by the cells suppresses the accumulation of excessive cAMP in the aggregation field. Propagating cAMP waves can be visualized by dark-field optics and time-lapse video

microscopy as rotating spirals or outward propagating concentric rings depending on the strain (Alcantara and Monk, 1974; Dormann et al., 1998; Durston, 1974; Gross et al., 1976; Tomchik and Devreotes, 1981, Siegert and Weijer, 1989). The geometry of propagating waves is analogous to the spatio-temopral pattern of chemical waves in the Belousov-Zhabotinski reaction.

Many experiments suggest that cAMP signaling also plays a major role during slug migration. Slugs have cAMP receptors, adenylyl cyclase and phosphodiesterase (Henderson, 1975; Rutherford et al., 1982) and have an overall tip to tail gradient of cAMP concentration (Brenner, 1977; Pan et al., 1974). Slug tips secrete cAMP and attract aggregation competent cells (Bonner, 1949; Rubin, 1976; Rubin and Robertson, 1975). Slugs placed on agar containing high concentrations of cAMP disaggregate (George, 1977; Nestle and Sussman, 1972; Wang and Schaap, 1985). cAMP is synthesized from ATP by the catalytic action of adenylyl cyclase. Three different adenylyl cyclases have been identified in *Dictyostelium.* Two of them, adenylyl cyclase A (ACA) and adenylyl cyclase B (ACB), are both active in the slug stage (Kim et al., 1998; Meima and Schaap, 1999b). Slug formation and slug movement can, however, occur in a mutant strain carrying a deletion in adenylyl cyclase A gene (*aca*A-PKA-C; Wang and Kuspa, 1997). Inhibition of adenylyl cyclase activation by caffeine results in the formation of multiple of new tips, suggesting that suppression of cAMP waves induces the formation of new tips in the prespore zone (Siegert and Weijer, 1993). There are four cAMP receptors known to be sequentially expressed during the developmental cycle of *D. discoideum* (Parent and Devreotes, 1996). Among those receptors, expression of *car3* and *car4* overlaps during slug stage. *car3* is expressed by all slug cells (Yu and Saxe III, 1996) while *car4* is preferentially expressed by tip cells and a *car4* disruptant has a reduced anterior prestalk zone (Louis et al., 1994). Direct observation of cell movement in migrating slugs showed that cell movement in the prespore zone is periodic, indicating that the movement might be directed by cAMP

waves (Durston and Vork, 1979; Durston et al., 1979; Siegert and Weijer, 1992). Consistent with the earlier finding of an transient increase in the cytosolic free calcium concentration, $[Ca^{++}]_i$, upon cAMP stimulation (Abe et al., 1988), pulses of $[Ca^{++}]_i$ with a period similar to the cAMP waves were detected (Cubitt et al., 1995). All these observations support the idea that cAMP waves organize slug behavior. Waves of cAMP are generated in the tip and are relayed to the remainder of the slug thus instructing individual cells to move chemotactically in the direction of tip migration. The result is coordinated movement of the slug.

The analysis of single cell movement in migrating slugs suggested that cAMP waves propagate as a three dimensional spiral (Abe et al., 1994; Siegert and Weijer, 1992). Prestalk cells in the anterior zone rotate around the long axis of the slug, i.e. they move perpendicular to the direction of slug migration, while prespore cells move straight forward in the direction of slug migration. These movements implicate the geometry of the propagating cAMP wave: a scroll wave in the prestalk zone and planar waves propagating from front to back in the prespore zone. Such a complex wave geometry could be explained by a difference in the excitability (i.e. the time constants of the proteins involved in cAMP signaling) between prestalk and prespore cells, with the prestalk zone being more excitable (Bretschneider et al., 1995; Steinbock et al., 1993). These cell types are known to have differences in the number and the type of cAMP receptors and the expression level of adenylyl cyclase and phosphodiesterase (Ginsburg and Kimmel, 1997; Hall et al., 1993; Saxe III et al., 1996; Wu et al., 1995; Yu and Saxe III, 1996). The difference was explicitly demonstrated by their different chemotactic behavior toward cAMP (Early et al., 1995). A lower excitability in the prespore zone could be due either to all prespore cells having a lower excitability in their response to the cAMP waves or to only anterior-like cells in the prespore zone responding to the waves (Bretschneider et al., 1995).

2.4 Slug Phototaxis

Slugs respond to environmental stimuli. When light is laterally irradiated, slugs immediately turn and migrate toward the light source by phototaxis. In the dark slugs show spontaneous turnings with a certain probability. This probability decreases during phototaxis due to the directed migration (Fisher et al., 1983). The phototaxis of the slug is very sensitive; white light with an intensity of 1 µW / m^2 is enough for a slug to orient itself towards the light source (Poff and Butler, 1974). Earlier studies demonstrated the key-role of prestalk cells in phototaxis (Francis, 1964; Häder and Burkart, 1983; Poff and Loomis, 1973). Phototactic turning is initiated exclusively at the tip (**Fig. 2a**). The irradiation of various regions of a slug with a small spot of light clearly showed that slugs sense light only in the anterior zone. Tip transplantation experiments between wild type and phototaxis negative mutants further showed that, if a wild type tip was grafted to the posterior fragment of a phototaxis deficient mutant, the mutant showed phototaxis, while a mutant tip grafted to the posterior fragment of a wild type slug resulted in blind slugs (Fisher et al., 1984). These experiments demonstrate that prestalk cells in the anterior zone sense the light and at the same time are responsible for phototactic turning.

Several results demonstrate that slug phototaxis involves a lens effect. First, when one side of the anterior zone was irradiated with vertical beams of light, slug turned away from the irradiated side (Francis, 1964; Häder and Burkart, 1983; Poff and Loomis, 1973). Second, when the slug was immersed in mineral oil to increase the refractive index, unilateral light would be expected to diverge rather than converge over the slug. In this case, slugs did not turn towards but turned away from the light source (Bonner and Whitfield, 1965). Third, when slugs were heavily stained with the prestalk-specific dye neutral red to absorb irradiated light and to stimulate only the irradiated side, the slug migrated away from the light source (Häder and Burkart, 1983; Wallraff and Wallraff, 1997). All these experiments indicate

that unilateral light refracted by the cylindrical surface of irradiated side (proximal side) is focused on to the other side (distal side). The slug turns away from this focused light and thus toward the light source.

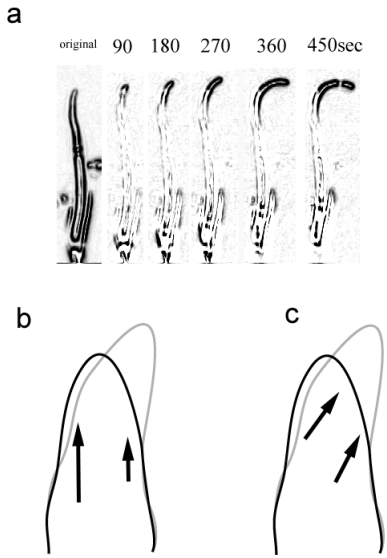

Figure 2. Slug Tip is Responsible for the Phototactic Turning.

(a) A montage of the phototactic turning of a slug. Light was irradiated from the right side of image. Images were taken at indicated times and slug image at time 0 (”original”) was subtracted from other time points. Slug turns from the tip. (b) Differential Speed Hypotheses. (c) Tip Activation / Inhibition Hypothesis. Black line: contour of slug anterior zone. Gray line: Contour after the turning. Arrows show the expected cell movement vector in each hypotheses.

2.4.1 Action spectrum

The action spectrum shows strong phototactic responses to wavelengths of 420, 440, 560 and 610 nm (Francis, 1964; Poff et al., 1973; Poff and Häder, 1984). A heme protein isolated from mitochondrial fraction is oxidized by light and has been characterized as the probable photoreceptor pigment (Poff and Butler, 1974; Poff et al., 1974). No study using a molecular genetic approach has been made to confirm the role of this photoreceptor pigment in phototaxis.

2.4.2 Single cell phototaxis

The simplest idea for the mechanism for phototaxis is that the phototaxis of slug cells collectively results in slug phototaxis. There have been many attempts to demonstrate phototaxis of single cells but these efforts were generally unsuccessful (Bonner and Whitfield, 1965; Francis, 1964; Samuel, 1961). One experiment showed that when a field of vegetative cells was irradiated with a spot of light, cells accumulated at the irradiated spot but the effective light intensity was limited to a very narrow range (Häder and Poff, 1979b). In addition, it was shown that cells accumulated or dispersed from a light spot depending on the absolute intensity of light. This result is not consistent with slug phototaxis which occurs over a broad range of light intensities (Hong et al., 1981; Poff and Häder, 1984). Further results question the hypothesis that a collective phototactic behavior of single cells underlies the phototactic behavior of slug. Prestalk cells dissociated from slugs do not phototax (Francis, 1964). The phototaxis of single vegetative cells is multidirectional, exhibiting 12 preferred direction towards the light source (Fisher et al., 1985). The action spectrum of the single cell photo-response (Poff and Häder, 1984) is different from that of slug phototaxis (Häder and Poff, 1979b; Häder and Poff, 1979c; Schlenkrich et al., 1995). Thus, the light-reaction of single cells is currently thought not to be directly related to the phototaxis of slugs.

2.4.3 Hypotheses of phototactic turning

How does a slug turn towards a light source? Since a concerted movement of cells drives slug migration, phototactic turning should be mediated by changes in cell movement within the anterior prestalk zone. In principle two types of change in cell movement could induce a turning of the slug; change of either cell speed or orientation of cell movement. Two hypotheses have been proposed to explain phototactic turning, based mostly on macroscopic studies of slug behavior.

The differential speed hypothesis suggests that the movement of cells distal to the light source (where the lens effect focuses the irradiated light: referred as distal cells in the following) is activated. The difference in cell speed between one side and the other bends the anterior zone towards light (**Fig. 2b**; Bonner, 1994; Poff and Loomis, 1973).

One of the candidates for activating cell movement is ammonia (NH_3). NH_3 is a gaseous metabolite that is produced through proteolysis and is emitted by slugs (Bonner, 1993). It acts as a repellent, based on the fact that NH_3 gas delivered next to the slug by a micropipet could the slug to turn away from the micropipet (Kosugi and Inouye, 1989). Light stimulates NH_3 emission and external application of NH_3 inhibits phototaxis (Bonner et al., 1988). NH_3 speeds up cell migration, stream formation (Bonner et al., 1986) and slug migration (Bonner et al., 1988). It may act by increasing the pH in an intracellular compartment (Kosugi and Inouye, 1989). From these facts, it was proposed that NH_3 emission on the side of the slug distal to the light source causes higher cell speed in that region, which then leads to a turn of the anterior zone toward the light source (Bonner, 1994; Bonner et al., 1988). The formation of steady gradient of NH_3 across the anterior zone, however, was questioned considering the high diffusion rate of NH_3 gas

(Fisher, 1991).

Another candidate for activating differential cell movement is the slime sheath. Comparison of the slime sheath of slugs migrating at different speeds demonstrated that the sheath thickness is constant regardless of the slug speed (Farnsworth and Loomis, 1974). This result was the reverse of the expected, in which, if the rate of synthesis is constant, faster movement of slugs should produce a thinner slime sheath. The unexpected result lead to a hypothesis that cell speed within slugs is dependent on the rate of slime sheath synthesis; faster synthesis allows faster movement (Poff and Loomis, 1973). In the case of phototaxis, the rate of slime sheath production should be higher on the side distal to the light source, which then increases the cell speed on that side and subsequently turns the anterior zone toward the light (Poff and Loomis, 1973). This conjecture has not yet been tested thoroughly.

If cell movement activity increases upon light irradiation, one possible consequence could be that the whole slug increases its speed. Several investigators have studied the change in the speed of the slug movement following light irradiation but the results were conflicting. Some studies reported that slugs do not change their speed upon light irradiation (Bonner and Whitfield, 1965; Raper, 1940; Smith et al., 1982), while the others reported an increase in slug speed (Kitami, 1982; Poff and Loomis, 1973). An alternative strategy was to follow cell movement during the turning of flat 2D slugs. The observation of spontaneous turning (not phototaxis) showed that, during turns, there was no change in cell movement activity. This result is not conclusive, however, for the geometry is different in 2D slugs and the mechanism of turning could be different. In addition, the mechanism of phototactic turning may not be the same as that of spontaneous turning.

An alternative model for phototactic turning is the tip activation / inhibition

hypothesis. This hypothesis was formulated based on an analogy to the coupled tip activation and tip inhibition process that generates the distinct tip-tail polarity of the slug (Fisher et al., 1984; MacWilliams and David, 1984; Meinhardt, 1983). It is assumed that light activates the formation of new tip proximal to the light source while inhibiting the tip formation on the distal side, thus orienting the cells to extend a new tip towards the light source (**Fig. 2c**; Fisher et al., 1984). In this case, the focused light on the distal side induces a tip inhibition signal and the proximal side is stimulated by a tip activation signal.

cAMP was proposed as a candidate for the tip activation signal based on the fact that the propagation of cAMP waves from the anterior tip organizes slug movement (Fisher *et. al.,* 1984). It was shown in earlier studies that light affects both the size and the temporal development of cell aggregates (Konijn and Raper, 1965; Konijn and Raper, 1966). Light also inhibits the aggregation of cells in a dose-dependent manner (Häder and Poff, 1979a). The transfer of slugs to substrates containing adenosine or caffeine, both cAMP signaling antagonists, impairs phototaxis (Darcy and Fisher, 1990). In addition, overexpression of a mutant regulatory subunit (Rm) of the cAMP-dependent protein kinase (PKA), which suppresses the stimulation by cAMP, also impairs slug phototaxis (Bonner and Williams, 1994). Finally, disruptants of the cAMP receptor *car3* or *car4* genes form migrating slugs but their phototaxis is impaired (Fisher, 2000). All these results indicate that the mechanism of phototaxis is tightly linked to cAMP signaling. However there has been no experimental evidence that showed an activation of cAMP waves on the proximal side nor a suppression of cAMP wave in the distal side during phototactic turning.

As candidates for the tip inhibitor signal, gaseous ammonia, adenosine and slug turning factor (STF) have been proposed (Fisher, 2000). Gaseous NH_3 is also a candidate for the cell speed increase signal as described above. Conversely, NH_3 attenuates cAMP signaling by a transient inhibition of

adenylyl cyclase (Schaap et al., 1995) and by antagonizing the cAMP receptor during aggregation phase (Siegert and Weijer, 1989; van Haastert, 1985; Williams et al., 1984). Adenosine, a product of cAMP hydrolysis by phospodiesterase and 5'-nucleotidase (Newell, 1983; Newell and Ross, 1982; Theibert and Devreotes, 1984), impairs phototaxis at a concentration comparable with the binding constant of adenosine receptors located at the cell surface (Darcy and Fisher, 1990; van Haastert, 1983). Since adenosine competitively antagonizes cAMP signaling system, it could have a role in suppressing tip formation and in generation of the prestalk zone (Schaap and Wang, 1986). Slug turning factor (STF), a non-volatile repellant of slug chemotaxis, also impairs phototaxis at high concentration in the agar substrate and its secretion is stimulated by light (Fisher et al., 1981). The detailed kinetics of STF secretion upon light stimulation are unknown. Whether the speed of STF emission is comparable to the time constants of slug turning has never been tested. Thus, the role of STF in slug turning is still under question. Both hypotheses, the differential speed hypothesis and the tip activation / inhibition hypothesis, are not mutually exclusive, since formation of the new tip could entrain the cells and possibly change the speed of the cells.

2.4.4 Pharmacological and genetic studies of phototaxis

Pharmacological and genetic investigations suggest that phototaxis involves intracellular signal transduction. Deficiencies in phototaxis induced by specific inhibitors or by the specific gene mutation have implicated the involvement of: G protein signaling (Darcy and Fisher, 1989), small GTP binding protein RasD (R. Insall, personal communication), the signaling protein glycogen synthesis kinase 3 (GSK3; Fisher, 2000), inositol polyphosphate signaling (Darcy and Fisher, 1989), extracellular Ca^{++}, ribosomal RNA of mitochondria (Wilczynska et al., 1997), cyclic GMP dynamics (Darcy et al., 1994a; Darcy et al., 1994b) and the regulation of

the architecture of filamentous actin network by actin binding proteins (Fisher et al., 1997; Stocker et al., 1999; Wallraff and Wallraff, 1997).

2.5 Questions

Although slug phototaxis has been extensively analyzed, major questions are still unanswered. There is no accepted hypothesis as to how light is sensed by a slug and how this signal is processed to induce phototactic behavior. It is still unknown how cells move within slugs in response to light irradiation. Mutations and drug treatments have revealed several proteins and signal transduction pathways involved in phototaxis. Most of these studies have been limited to the analysis of slug trails and hence they only provide information on the orientation and the accuracy of phototaxis. This has been efficient for screening mutants to identify the components and pathways involved in phototaxis but does not provide direct clues about the mechanism of phototaxis.

Although several hypotheses have been proposed for the mechanism of phototactic turning, none of these hypotheses has been experimentally verified. For these reasons, slug behavior and the corresponding cell behavior before and after light irradiation were analyzed in detail with digital video microscopy. Many of the results depended on newly developed methods. Optical conditions using infrared illumination were set up to permit observation without interfering with the phototactic reaction of slugs and cells. Experiments were designed to clarify the relationship between slug movement and cell movement within a slug. Computer programs were written to analyze digital image sequences for measuring slug and cell behavior and morphology. The results revealed previously unknown dynamic features of slug and cell behavior during phototaxis. This approach can be further used to analyze phototaxis mutants to link the regulation of phototaxis at the molecular level to the cellular and multicellular level.

3 Materials and Methods

3.1 Cell culture and slug preparation

Dictyostelium discoideum strain NC-4 grown on bacteria *Klebsiella aerogenes* was used in all experiments. All cell culture and experiments were done at 22 ±1°C. Cells were grown on nutrient SB agar plates (Sussman, 1987) for 48 ± 4 hours, washed by centrifugation with filter-purified water (>18W; Milli-Q Plus water purification system, Millipore GmbH, Eschborn, Germany) for slug preparation, or with KK_2 buffer (20 mM K_2HPO_4/KH_2PO_4, pH6.8) for cell preparation. For slug preparations, a small drop of concentrated cells (approximately 10^8 cells/ml) was placed on non-nutrient water agar (Difco bacto-agar; 0.8% w/v) for development. The agar plates were enclosed in a metal box to keep them absolutely dark during incubation. Slugs formed after approximately 24 hours and started to migrate away from the drop. For cell preparations, 1 ml of cell suspension ($5x10^6$ cells/ml KK_2 buffer) was spread on a KK_2 agar plate (0.8% w/v), kept still for 30 minutes for adhesion of the cells to the substrate and then the supernatant was decanted. *car1-/car3-* cell line RI-9 (Insall et al., 1994) was grown in HL5 medium (Watts and Ashworth, 1970) supplemented with 0.1% geneticin (ICN Biomedicals Inc., Aurora, OH. USA) and 1% penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO. USA) and harvested at the late log phase of development. Further cell preparation followed the same procedure as wild type NC-4.

3.2 Dark chamber

A dark chamber was designed and constructed as illustrated in **Figure 3.** Agar plates (85 mm x 15 mm) with migrating slugs or cells were fitted into the chamber which was then tightly closed to avoid changes in humidity. Slugs or cells were observed from the ventral side. The bottom and the lid of the dark chamber were equipped with long pass filters (half-maximum at

695 nm; Andover Corporation, Salem, NH. USA) thus allowing observation of slugs under illumination well beyond the phototactic action spectrum (Francis, 1964; Poff and Häder, 1984).

3.3 Microscopy for slug movement analysis

Dark field-optics (Gross et al., 1976) with modifications (Siegert and Weijer, 1989) was used for imaging slug migration. Slugs in the dark chamber were illuminated from below and their migration was monitored from above by a CCD camera (VC-2512, Sanyo, Osaka, Japan) equipped with a 30-70 mm Tokina Zoom objective and a 2x zoom lens. The dark-field illumination allowed the observation of slug trails and tip up-down motion (see Results). Slug trails cannot be observed in normal bright field illumination and the conventional method for obtaining slug trails has been a complicated procedure including drying and staining with Coomassie Blue (Fisher et al., 1981). Thus, the use of dark field illumination facilitated the measurement of slime trails. Using an image processor board (AFG, Imaging Technology) time lapse video sequences were digitized, contrast enhanced, averaged (32 frames) to reduce noise and then stored on a laser-disc recorder (LVR-4000AP, Sony, Tokyo, Japan.) or computer hard disk for further analysis.

3.4 Phototactic stimulation

Light for phototaxis was sent through pinholes (1.5 mm ∅) with shutters in the side of the dark chamber (**Fig. 3**), which were located at approximately 4 mm above the agar surface to eliminate the shading effect by the other slugs (Smith et al., 1982). The light for phototactic stimulation was passed through a glass fiber (Flexilux HLU90, Schölly Fiberoptik, Denzlingen, Germany) to eliminate heat effects (thermotaxis). Several experiments were performed with light filtered by a blue filter (short pass filter, half-maximum at 600 nm; Andover Corporation, Salem, NH. USA) and similar results were

obtained. To observe slug motion images were acquired every 45 seconds. Usually the slugs were allowed to migrate for 1-2 hrs in the dark before the pinhole shutter was opened and light irradiation started. In some experiments the pinhole shutter was closed after several hours of migration to check the reversibility of light effects.

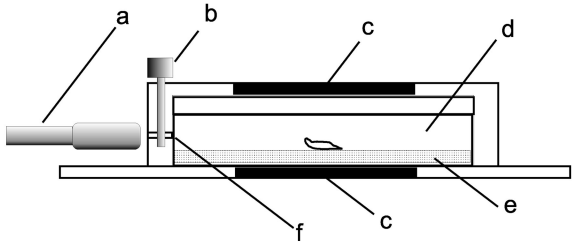

Figure 3. Dark Chamber.

Side view of dark chamber designed for the analysis of phototaxis. A culture dish (d) containing slugs migrating on agar substrate (e) was fitted inside. The chamber had windows with long-pass filter at its top and bottom (c) to allow observation without affecting the phototactic response of the slug. A pinhole (f) with shutter (b) was on the side wall to initiate phototaxis by light (a) at desired time point.

3.5 Analysis of phototactic behavior

Figure 4 shows the definition of the slug body. To analyze the movement of slugs, the x-y co-ordinates of the slug tip and tail were measured by

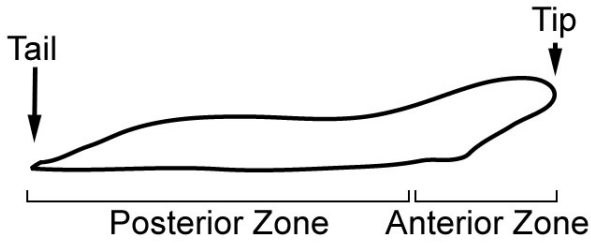

Figure 4. Body parts of slug.

placing a screen cursor on the corresponding points in each frame of a time series. A custom C program was written to control (1) the laser disc recorder, (2) the image capture board (3) and to read out the x-y coordinates of cursor position and store them in a data file. The data files were imported in the software IgorPro (version 3.14, WaveMetrics Inc, Lake Oswego, OR. USA) and calculations were done by custom written macro programs.

The speed of the tip and tail were calculated from consecutive x-y coordinates in 45 seconds intervals. For each time point the displacement of the tip or the tail within ±225 seconds was calculated and considered as the speed at that time point. The formula used to calculate the slug speed at time point t0 was as follows:

$$\text{Slug Speed} = \{ (X_{t+5} - X_{t-5})^2 + (Y_{t+5} - Y_{t-5})^2 \}^{0.5} / T_{int} / 10$$

where (X_{t+5}, Y_{t+5}) is the X-Y coordinate of the tip 5 frames after the time t0, (X_{t-5}, Y_{t-5}) is the X-Y coordinate of the tip 5 frames before time t0 and T_{int} is the time interval between successive frames in seconds. Calculated values

were then converted into mm/hr.

The periodicity of the up and down motion of the tip ("blinking", see Results) was measured by averaging the intensity of 5 x 5 pixels at the tip. This procedure was included in the C program for measuring the x-y coordinates. Under dark field illumination, it was very easy to determine both the anterior and the posterior end of the slug.

The term "slug length" was defined as the distance between the tip and the tail and could be calculated from their x-y coordinates. The slug length at successive time point was calculated as follows:

$$\text{Slug Length} = \{(X_{tail} - X_{tip})^2 + (Y_{tail} - Y_{tip})^2 \}^{0.5}$$

where (X_{tip}, Y_{tip}) is the X-Y coordinate of the tip and (X_{tail}, Y_{tail}) is the X-Y coordinate of the tail. Calculated values were converted into mm.

The orientation of slug was defined as an angle in radian clockwise between the vertical axis of the image frame and the slug long axis (**Fig.5a**). The long axis was defined as a straight line drawn between the tip and the tail. For example, when the long axis of a slug is aligned perfectly with the vertical axis of the image frame and when the tip of the slug was heading toward the top of the image frame, slug orientation was 0 rad. When the tip was heading toward the bottom of the frame, the slug orientation was π rad. The slug orientation was calculated as following:

$$\underline{S}\text{lug } \underline{O}\text{rientation (SO) [rad]} = \arccos \{ (Y_{tail} - Y_{tip}) / \{ (X_{tail} - X_{tip})^2 + (Y_{tail} - Y_{tip})^2 \}^{0.5} \}$$

where (X_{tip}, Y_{tip}) is the X-Y coordinate of the tip and (X_{tail}, Y_{tail}) is the X-Y coordinate of the tail. When $X_{tail} > X_{tip}$, slug orientation was further calculated to expand the range of orientation to 2π [rad];

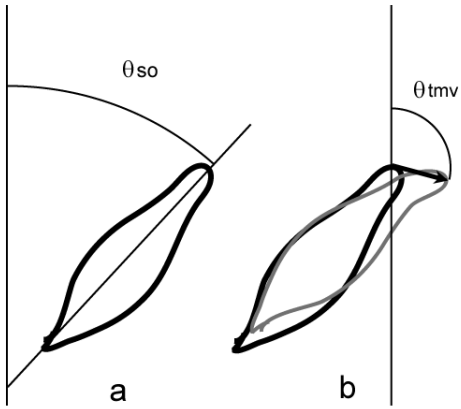

Figure. 5 Slug Orientation (a; q_{so}) and Tip Movement Vector (b; q_{tmv}).

SO = 2π - SO.

By calculating the orientation in each frame of an time series, dynamics of slug orientation could be obtained.

A vector of tip movement was defined as the angle in radian clockwise between the vertical axis of the image frame and the orientation of the tip movement (**Fig. 5b**). It represents the direction of the tip movement in successive time points. The tip movement vector at time t0 was calculated as follows:

Tip Movement Vector (TMV) [rad] = $\arccos \{ (Y_{t-5} - Y_{t-5}) / \{ (X_{t+5} - X_{t-5})^2 + (Y_{t-5} - Y_{t-5})^2 \}^{0.5} \}$

where (X_{t+5}, Y_{t+5}) is the X-Y coordinate of the tip 5 frames after time t0 and (X_{t-5}, Y_{t-5}) is the X-Y coordinate of the tip 5 frames before time t0. When $X_{t+5} < X_{t-5}$, slug orientation was further calculated as following to expand the range of orientation to 2π [rad];

$TMV = 2\pi - TMV$.

3.6 Ammonia experiments

A small plastic culture dish (35 mm x 10 mm) with slugs migrating on water agar was placed inside a large plastic culture dish (85 mm x 15 mm) containing 20 ml of ammonia generating solution and incubated for 3 hours at 22 ±1°C in the dark. The duration of incubation was long enough to estimate slug speed from the slime trails (approximately 1 ~ 3 mm; data not shown). An ammonia generating solution was prepared from 0.5M NaOH and various concentrations of NH_4Cl up to 1 mM as described in (Bonner et al., 1988). Slug trails were visualized using dark field illumination and digitally stored before and after incubation. ScionImage (Version beta3, Scion Corp., Frederick, MD. USA) image processing software was used to trace slime trails manually and to measure their length to obtain slug speed by the following formula:

Slug Speed [mm/hr] = Length of the slime trail [mm] / Duration of treatment [hr].

3.7 Effect of flickering light source on slug migration

Agar plates with migrating slugs were placed in a paper box lined inside

with black paper. The space inside the box was divided in a small compartment for the light bulb and a larger one for the agar plates. The wall between both compartments was made of black paper and had a slit in the middle for light irradiation. Three agar plates with slugs were placed in the paper box, one of the plates was wrapped with aluminum foil as a control. The box was closed, and wrapped with aluminum foil to avoid light leaking from the outside. The light bulb was turned on and off by a computer controlled timer board (ME-14A, Meihaus Electronic GmbH, Puchheim Germany) and a C program was written to control the flickering interval of the light. The duration of on/off periods was equal resulting in the same total duration of irradiation regardless of the flickering interval. Images of slug trails were saved on hard disk before and after 24 ± 4 hours of flickering light treatment at 22 ±1°C. Total trail length and net displacement of the slug trails was measured using ScionImage as follows. The slug trails before and after the experiment was compared to define the starting point. Total trail length was measured by manually tracing the slug trails (**Fig. 6**). Net displacement was measured by drawing a straight line between the start and the end point. For each flickering interval more than ten trails were measured. To compare the randomness of migration with variable flickering intervals, the efficiency of phototaxis was calculated as follows.

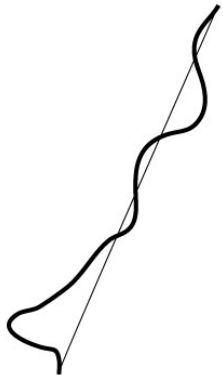

Figure 6. Measurement of Efficiency of Phototaxis.

Thick line represents the total trail length and thin line represents the net displacement.

Efficiency of Phototaxis = Net displacement [mm] / Total length of the slug trail [mm].

3.8 DiR labeling of slug cells and its detection

2 ~ 5% of the cells of a slug were labeled with DiR ($DiIC_{18}(7)$, Molecular Probes, Eugene OR., USA) by incubating 10^6 washed cells in 1 ml DiR solution (25 µg DiR/ml KK_2 buffer, 200 mM K_2HPO_4 and KH_2PO_4, pH6.8) for thirty minutes in the dark. Then the stained cells were washed twice, mixed with unstained cells and allowed to form slugs as described. Slugs with 100% DiR labeled cells showed normal phototaxis (data not shown). Excitation and emission wavelength of DiR were 750nm and 780nm respectively, well above the range of light inducing phototaxis (Francis, 1964; Poff and Häder, 1984). The DiR signal was detected with the use of a specific filter set (XF49, Omega Optical, Brattleboro, VT., USA) inserted into the light path between sample and a xenon lamp. Digital images were acquired with a high sensitivity cooled CCD camera (C4880, Hamamatsu Photonics, Hamamatsu, Japan) controlled by HiPic software (Ver.5.0.0 Hamamatsu Photonics). An automatic shutter in the illumination path was synchronized with the image acquisition so that the slugs were exposed to the excitation light only during image acquisition (~ 2 sec). This was done by including a custom made dynamic link library file to the HiPic software.

3.9 Analysis of cell movement during phototactic turning

Images of DiR stained slug cells were captured every 30s in the dark and during phototaxis. Light irradiation for phototaxis was done as described in “Analysis of phototactic slug behavior“. Cell movement and corresponding changes in slug shape were analyzed in two ways using the software Photoshop (Adobe Systems Inc. San Jose, CA., USA) and custom macro

programs written for ScionImage and IgorPro software. Cell positions in each frame were marked by dots manually. This procedure was required since the intensity of fluorescence signal differed from cell to cell. Therefore the automated thresholding which is required for automated tracking did not work. Then the x-y coordinates of the dots were measured and saved by a custom program written in ScionImage. Further analysis was done with IgorPro. In the wire-frame analysis, the geometry of the relative positioning was made visible in each frame by drawing lines between the same group of cells (see **Fig. 26**). At the same time, the contour of the slug was traced manually to analyze the relationship between relative movement of the cells and the changes in the shape of the slug. In the slug cell velocity analysis, cell velocity was calculated by the following formula:

$$\text{Cell Velocity} = \{ (X_{t+1} - X_t)^2 + (Y_{t+1} - Y_t)^2 \}^{0.5} / T_{int}$$

where (X_t, Y_t) is the X-Y coordinate at time t, (X_{t+1}, Y_{t+1}) is the X-Y coordinate at time t+1 and T_{int} is the time interval between successive frames. Then lines between positions (X_t, Y_t) and (X_{t+1}, Y_{t+1}) were drawn and overlaid on an image of slug contours both at time t and t+1. Calculated cell velocities (in µm/sec) were tagged to these displacement lines (see **Fig. 27**).

3.10 Analysis of the light effect on cell movement within flattened slug

To restrict three-dimensional movement, slugs with DiR labeled cells were overlaid with agar as described above. Then the plate was placed in the dark chamber with upper side window closed by a metal plate. After 0.5 hours incubation in the dark on the inverted microscope, recording of the image sequence was started. In dark conditions, images of DiR signals were detected by excitation light delivered from the bottom of the sample. To irradiate with light within the action spectrum of the slug or cells, the metal

plate in the upper side was replaced with a short-pass blue filter (**Fig. 7;** half-maximum at 600nm; 600FL07-50s, Andover Corp.). Irradiation was done either by irradiating the whole slug (overall irradiation) or only part of the slug from above (spot irradiation). A light spot was made by narrowing the aperture of condenser. DiR stained cells were manually tracked and analyzed as described in “Analysis of cell movement during phototactic turning”. In the case of spot irradiation, cell tracks were visualized by a custom program written for ScionImage with the following algorithm. After adding a masking value to the image, frame at t_{np} was overlaid to the frame at t_{n+1} resulting a processed frame $t_{(n+1)p}$. The addition of masking value darkened the image frame at t_{np}. Repeating of this procedure with successive frames has resulted in cell tracks like a comet tail, with earlier positions being darker. If the masking value was high, cell tracks became short, representing cell tracks during a short time. Smaller masking value allowed visualization of longer tracks but the tracks overlapped and single cell tracks became undistinguishable.

3.11 Microscopy for cell movement analysis during aggregation phase

Cell movement was observed with a microscope (Axiovert 100TV, Carl Zeiss GmbH, Jena, Germany) equipped with 10x and 20x objectives (Plan-Neofluor, Carl Zeiss GmbH). The illumination light was filtered with a near infrared short pass filter (half-maximum at 800nm; 800FL07-50s, Andover Corporation, Salem, NH. USA) in all experiments. Digital images were acquired with a high sensitivity cooled CCD camera (C4880, Hamamatsu Photonics) controlled by HiPic software (Hamamatsu Photonics).

3.12 Effect of light in early aggregation phase

Plates with cells were kept in the dark and then transferred to the dark chamber at various times during aggregation and observed with phase contrast microscopy. Time lapse images were acquired every 20 seconds. To irradiate with light within the action spectrum, the red filter of the upper side of the filter was replaced with short-pass blue filter (**Fig. 7**; half-maximum at 600nm; 600FL07-50s, Andover Corp.). Analysis of the cell movement activity was done as follows, with custom programs written for

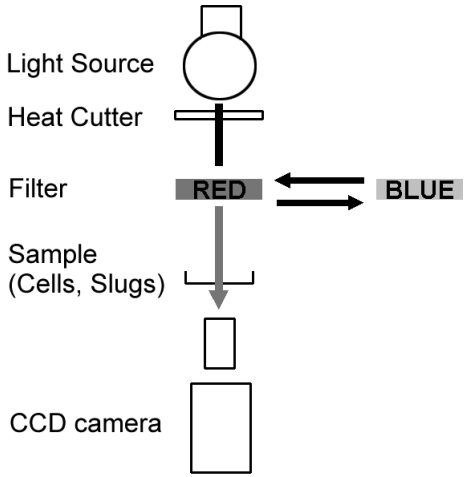

Figure 7. Method of Light Stimulation.

Red filter (or metal plate in the DiR slug experiment) was inserted to the illumination light path for the recording of the cell movement in dark. Red filter (or metal plate) was exchanged with blue filter for light irradiation. Cells could be observed continuously in the dark and after light stimulation.

ScionImage and IgorPro. First, a background image was subtracted from the sequence to reduce potential noise. Then image *t* (**Fig. 8a**) was subtracted from image *t+1* (**Fig. 8b**). This resulted in an image containing positive or negative pixel values of the cell area that protruded or retracted during the time interval of 20 seconds (**Fig. 8c**). By examining the subtracted image, ranges of the gray value that contained the information on pseudopod activity could be defined (**Fig. 8c** arrow and arrow head; **Fig. 8d**). Histograms of the gray values of each subtracted image were calculated (**Fig. 8d**) and the values were exported to software IgorPro. The number of pixels within the range of gray value, representing pseudopod activity, was calculated as the movement activity of the cells. Approximately two hundred cells would be measured at the same time.

3.13 Effect of light in late aggregation phase

Spores were placed in the center of a growing bacteria lawn on SB agar plate. Amoebae germinated from the spores and eventually formed an enlarging plaque with large cell streams at the edge of the plaque. Recording of dark-field waves in streams was started after the plate was enclosed in the dark chamber and adapted to dark conditions for 30 minutes. To initiate with light irradiation, the red filter of the upper side of the filter was replaced with short-pass blue filter (**Fig. 7**; 600FL07-50s, Andover Corp.). Light irradiation was restricted to a local spot within a stream under the illumination field of microscope. The effect of light irradiation was analyzed by time-space plots as described in detail in elsewhere (Siegert and Weijer, 1989).

3.14 Effect of light on cell-cell signaling in slugs

Plates with cells were kept in the dark for 1 hour after washing off bacteria. The anterior tip of a slug from a plate prepared a day before was dissected

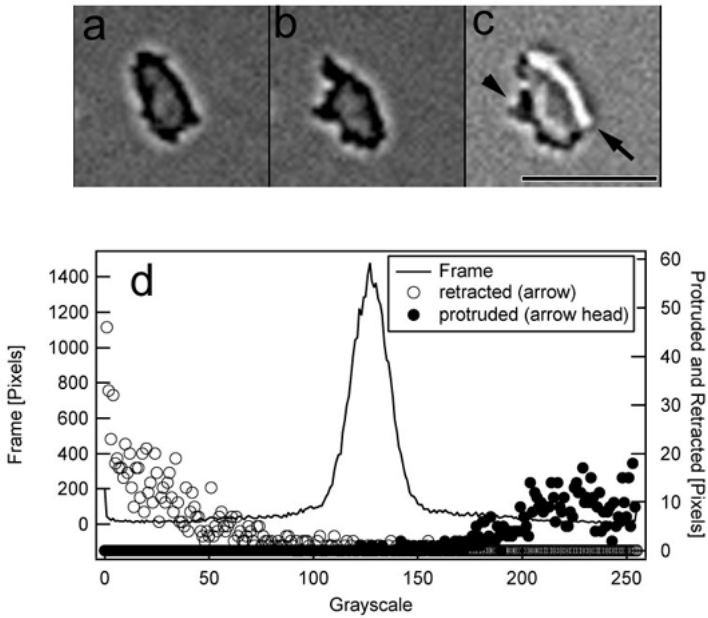

Figure 8. Method of the Cell Activity Measurement.

(a) Cell at time point *t*. (b) Cell at time point *t+1*. (c) Image calculation (image $_{t}$ – image $_{t+1}$ + 128). The arrow indicates retracted area. The arrow head indicates protruded area. (d) The gray value histograms of these areas and that of the full frame were measured. Cell movement activity was calculated by an integration of all pixels with gray values between 0~50 and 200~256. Note that the scales are different for the specific areas and the full frame. Bar=10μm.

and gently placed in the field of migrating cells. An agar film (2% w/v KK_2 buffer) was laid over the tip fragment to suppress its three dimensional movement (**Fig. 9**). The plate was then transferred to the dark chamber and images were recorded every thirty seconds. The slug tip attracted cells after transfer to the dark chamber. This effect decayed within ca. 30 minutes and was presumably due to light stimulation during tip cutting and transfer. After 50~60 minutes, cell movement became random and light effects could be studied. To irradiate with light within the action spectrum of the slug or cells, the red filter of the upper side of the filter was replaced with short-pass blue filter (**Fig. 7**; 600FL07-50s, Andover Corp.). Cell movement was analyzed during 40 minutes before and 20 minutes after light irradiation using ScionImage software and by manually tracking the cells. First, dots at the position of the each cells were drawn manually in the frames. Then the X-Y coordinates of the cell were obtained by a custom program written for ScionImage. At the same time, the program overlaid the cell tracks to the original image by connecting the consecutive position of the cells to analyze the orientation of cell movement. RI-9 cells were assayed for their chemotactic ability by two different methods. In the first assay, cell movement towards a glass needle filled with cAMP (1~100 µM) or folic acid (100 µM ~ 1 mM) was analyzed. In a second assay, dense drops of wild type or mutant cells (10^8 cells/ml, ∅ 1 ± 0.3 mm) were placed on chemoattractant containing agar substrate (100 µM cAMP or 1 mM folic acid in 0.8% agar / KK_2 buffer). Outward directed gradients of the chemoattractant were formed from the cell spots by hydrolysis of cAMP or folic acid. Outward migration was used to assess chemotaxis. Both assays indicated that RI-9 cells did not acquire chemotactic ability to cAMP until 4.5 hours after the starvation. Thus, RI-9 cells were used 0.5 ~ 2 hrs after starvation to test if the substance diffusing from the slug tip was cAMP. RI-9 cells did show chemotaxis toward folic acid (100 µM ~ 1 mM) within the first 4.5 hours after starvation, indicating that their chemotaxis deficiency was specific to cAMP.

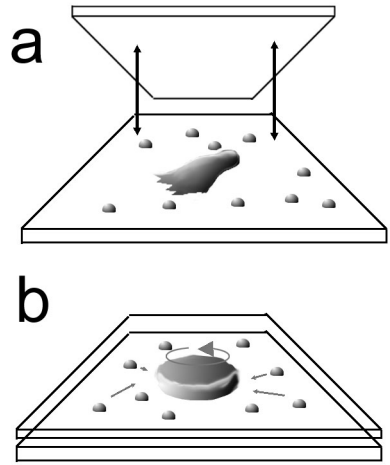

Figure 9. Method of Tip Assay.

(a) Slug tip was dissected and transplanted in the field of pre-aggregation cells. (b) Slug became disk-shaped and started rotation (large arrow) after the agar-overlay. Diffusion of cAMP from the slug tip attracted pre-aggregation cells (small arrows). Light effect was tested by the behavior of pre-aggregation cells.

4 Results

The coordinated movement of tens of thousands of cells drives slug migration. Accordingly, phototactic turning most likely involves changes in cell movement within the slug. To detect such changes, behavior on two different scales, namely, slug movement and cell movement within slugs, was analyzed and correlated.

4.1 Slug movement in dark and during phototaxis

There have been many reports on slug phototaxis. The analyses were mainly focused on the orientation of the slug trails left behind by migration; this was informative in deriving the accuracy of phototaxis and changes in the average speeds. However, details of how the light gradient affects cells, how cells move to turn the slug tip toward the light source and how slugs change their behavior could not be resolved from these macroscopic studies since slug trails were the result of several hours or days of slug migration. To understand slug behavior during phototaxis, detailed analyses with higher temporal and spatial resolution were done. The following sections describe the results of these analyses. Slug motility was observed using infrared illumination in order not to affect the phototactic behavior of the slug. Video microscopy and the analytical processing of digital image sequences enabled continuous analysis (time resolution = 45 sec) for long periods (~12 hrs) of single slugs in the dark and after phototactic stimulation. Measured parameters of slug behavior included slug speed, frequency of up-down motion of the tip (see below), slug morphology and orientation of both the tip movement and slug long axis.

4.1.1 Speed and up-down motion of slug tip

To analyze slug motility in detail for long periods, a dark chamber was constructed and dark-field illumination was used. The dark chamber was designed with two long-pass infrared filters on top and bottom for observations (**Fig. 3**). A hole with a shutter on the side wall permitted irradiation with light for phototactic stimulation while recording slug behavior. This enabled the continuous observation of slug migration up to 12 hours in the dark, during the initial turning towards light source and during phototactic migration.

Migrating slugs demonstrate a periodic up-and-down motion of the tip during migration in the dark. This up-down motion has been reported previously and described as serpentine arching movement (Breen et al., 1987; Inouye and Takeuchi, 1979), but this is the first time that this behavior has been analyzed quantitatively for long periods of time without affecting the phototactic behavior. Under dark field microscopy, periodic up-down motion of the tip could be observed as a periodic bright flash of light at the tip (**Fig. 10a**), which resulted from the scattering of irradiating light when the tip of the slug landed on the agar surface. This periodic flashing is referred as "blinking". To analyze the up-down motion of the tip quantitatively, the change in light intensity was measured over time. Each blink or landing of the tip lasted about 225 seconds. The up-down motion was highly periodic with a period length of 13.5 ± 4.0 min (n=11). The speed of the slug tip also changed periodically and was tightly coupled to the up-down motion of the tip (**Fig. 10b**). The slug slowed down when the tip landed on the agar surface and increased in speed after the tip had lifted off the agar. Forward displacement of the tip occurred when the tip was lifted. The mean speed of the tip was 1.33 ± 0.19 mm/hr (n=11). Speed was also measured at the posterior end of the slug (tail). The tip and tail speed both oscillated but there was no apparent phase relationship with the tip speed (**Fig. 11**).

Phototaxis was induced by opening a pinhole shutter on one side of the dark chamber and irradiating the slug unilaterally with white light. Within a few

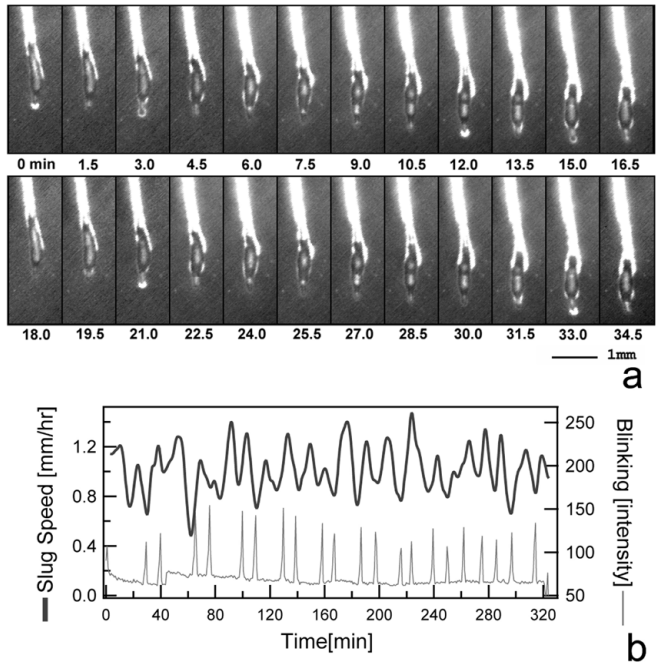

Fig. 10. Slug Tip Speed and Up-down Motion is Coupled.

(a) A sequence of slug migration in the dark. The slug was migrating in the dark chamber without irradiation of active light, from top to the bottom of the frames. Time interval between frames is 90 seconds. Whiteness fringing the tip corresponds to the degree of light scattering. White track extending upward from the slug is slug slime trail. Slug tip was lowered and attached to the agar substrate at the frames indicated with time points 0, 12, 21, 33 minutes, revealed by the blinking of the tip. Bar = 1 mm. (b) Slug speed (thick line) and light intensity (thin line) measured at the tip. Tip periodically landed on the agar substrate, shown by the periodic increase of the light intensity. Speed at the tip reduced when the tip attached to the substrate.

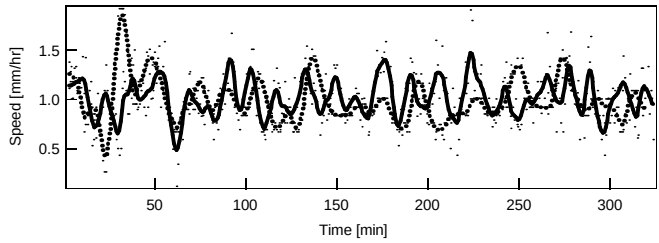

Figure 11. Relationship Between Tip and Tail Speed in the Dark.

Speed at the tip (black line) and tail (dotted line) are plotted against time.

minutes slugs changed their direction of migration towards the light source (**Fig. 12a**). Video analysis showed that the slug turning started at the tip as has been reported earlier (**Fig. 12a**; Francis, 1964; Poff and Loomis, 1973). There were dramatic changes in slug behavior accompanying phototaxis. After phototactic stimulation the blinking periodicity was more than doubled to 29.7 ± 10.1 min (n=6; **Fig. 12b**), while the duration of tip landing remained the same. At the same time the speed of the slug tip increased approximately 50% to 2.05 ± 0.32 mm/hr (n=6). These pronounced effects were both completely reversible. When the light was turned off, the speed of the slug tip and the blinking period decreased to the level before light irradiation (**Fig. 13**).

To determine whether the measured speed at the tip reflected the overall speed of the slug, slug speed was also measured at the tail. Measurements clearly showed that the speed at the tail increased as well, confirming the overall increase in slug speed (**Fig. 14**). As in slugs migrating in dark, there was no clear phase relationship between the oscillating tip and tail speed during phototaxis.

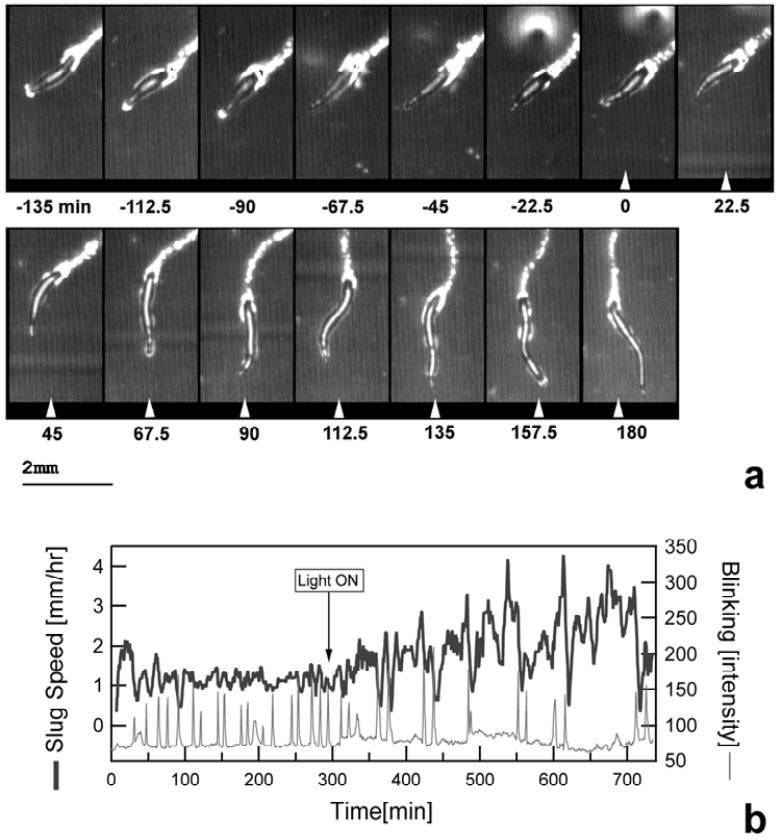

Figure 12. Light Increase the Slug speed and Decreases the Frequency of Up-Down Motion.

(a). A sequence slug migration before and during the unilateral light irradiation. Time interval between the frames is 22.5 minutes. At the frame indicated 0 minute, irradiation of the active light was initiated. Light direction is indicated by white arrowhead. Slug changed its shape from short and straight to long and twisted. Bar = 2mm. (b). Slug speed (thick line) and light intensity (thin line) measured at the tip. Slug increased its speed and frequency of up-down motion after light irradiation.

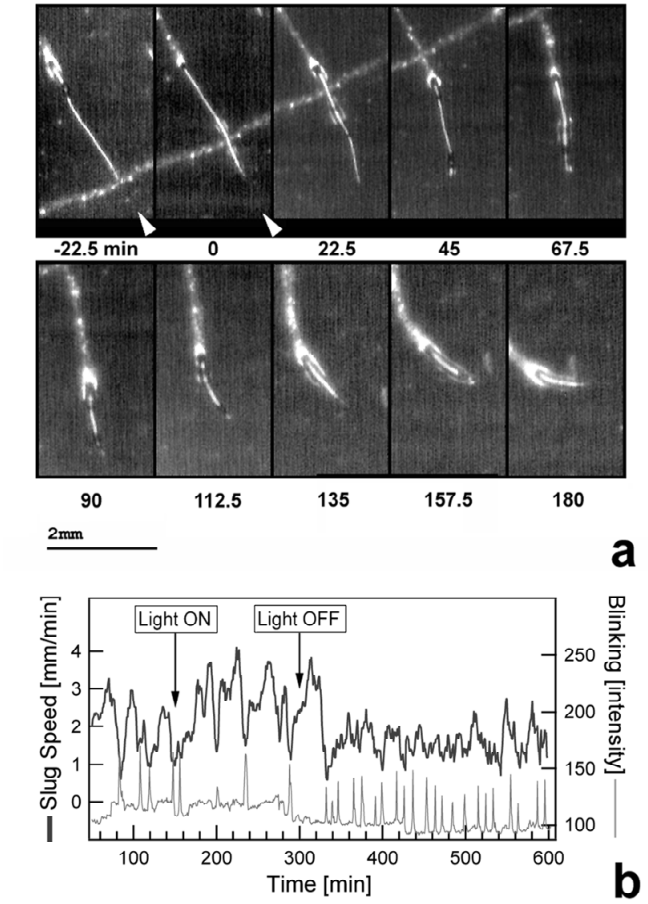

Figure 13. Reversibility of the Light effect.

(a). A sequence of slug migration during and after stopping the irradiation of unilateral light. Time interval between the frames is 22.5 minutes. Unilateral light was irradiated from the direction in left-bottom corner of each frame. At the frame indicated 0 minute, irradiation of unilateral light was stopped. Slug shortened its length after stopping the irradiation. Bar=2mm. (b). Slug speed (thick line) and light intensity (thin line) measured at the tip. Slug decreased its speed and frequency of up-down motion after the light was turned off.

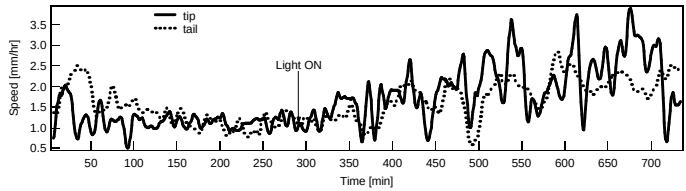

Figure 14. Relationship Between Tip and Tail Speed during Phototaxis.

Speed at the tip (black line) and tail (dotted line) are plotted against time.

4.1.2 Morphology

In addition to changes in movement speed, light irradiation also triggered distinct morphological changes in slugs. In the dark, the anterior zone of a slug formed a narrow nipple which could be easily distinguished from the slug body (**Fig. 15**; see also **Fig. 19**). The overall shape of the slug was straight and slug movement was also more or less straight. After light irradiation slugs showed significant changes in shape (**Fig. 15**; see also **Fig. 19**); the narrow nipple expanded and the tip became a smooth extension of the slug body. The slugs became longer (**Fig. 16**) and showed serpentine movement, in which the tip swung laterally to both sides (**Fig. 17**). Slug length increased up to 100% and the slugs clearly became thinner (n=8). Serpentine movement was quantified by calculating the vector of tip movement at 45 sec interval (**Fig. 18**). In the dark, the tip movement vector fluctuated slightly to both sides of the slug long axis. There was no apparent periodicity in this fluctuation. After light was turned on, the tip vector indicated a clear turning by 10 min after the irradiation. The change in the long axis orientation was more gradual than the change in the tip vector. The tip vector aligned with light axis approximately 40 min after light irradiation, while the turning of the long axis settled after approximately 90 min. This difference

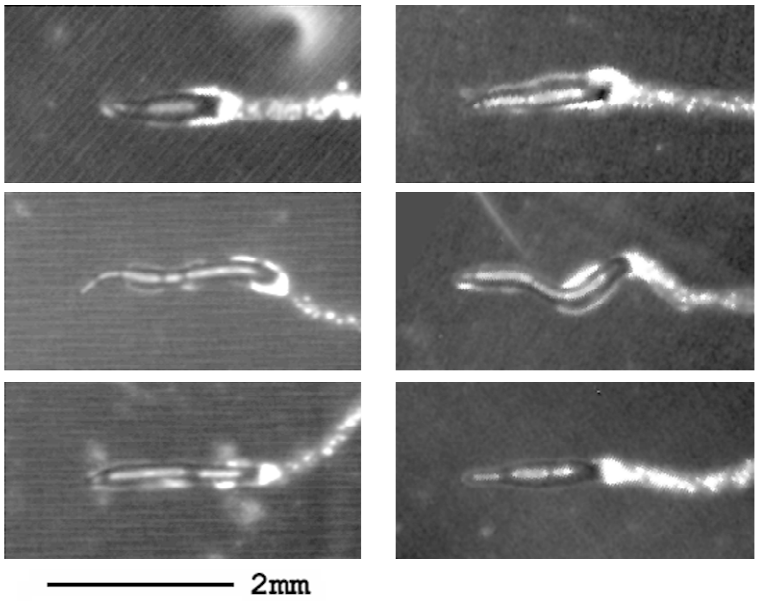

Figure 15. Effect of Light Irradiation on the Slug Morphology.

A gallery of morphological change induced by light irradiation. Each column shows dynamics of single slug. First row shows slug shape in dark. Note that slug tip is tapered. Second row shows slug shape after 150 minutes of light irradiation. Slug become longer and twisted upon light irradiation. Taper at the slug neck diminished and tip became a smooth extension from the slug body. Third row shows slug shape 150 minutes after stopping the light irradiation. Light effect on slug shape was reversible. Bar = 2mm.

was a simple reflection of the fact that the slug turning always starts from tip. Consistent with the observed serpentine movement, tip vector fluctuated periodically during phototaxis with an increased amplitude compared to that during dark migration (**Fig. 18**). All these shape changes were readily reversible after shutting the light off: the length decreased, the lateral oscillation of the tip ceased and the shape became straight as it was before irradiation (**Fig. 15, 16**).

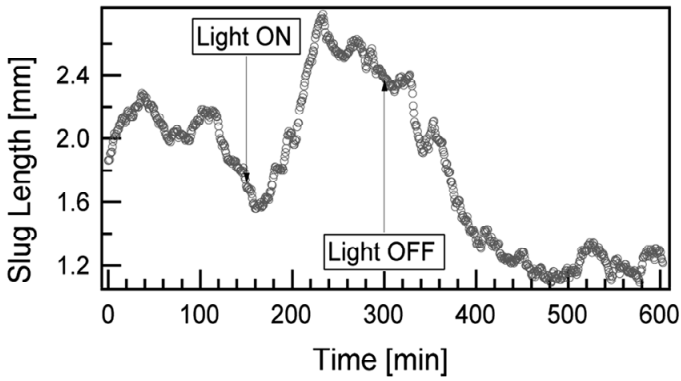

Figure 16. Light Increases the Slug Length.

Unilateral light was turned on and off at the indicated time points

To investigate the light induced elongation in more detail, video sequences were analyzed. Following light irradiation, a slug started to change its shape from the tip. The original cone shape of the tip became sharper, its diameter became thinner and it lengthened (**Fig. 19** Light ON 27~42 min). Turning initiated at 27 min (tip was bent to the left) suggesting that the turning was associated with this elongation process. The shape change at the tip was subsequently followed by shape changes in the posterior zone, such that it decreased its diameter and increased its length (**Fig. 19** Light ON 57~333min). After turning the light off, the diameter of the whole slug body gradually became thicker and then finally resumed its original shape (**Fig. 19** Light OFF 81~154min). A similar process of elongation and shortening was observed in all slug sequences (n=8).

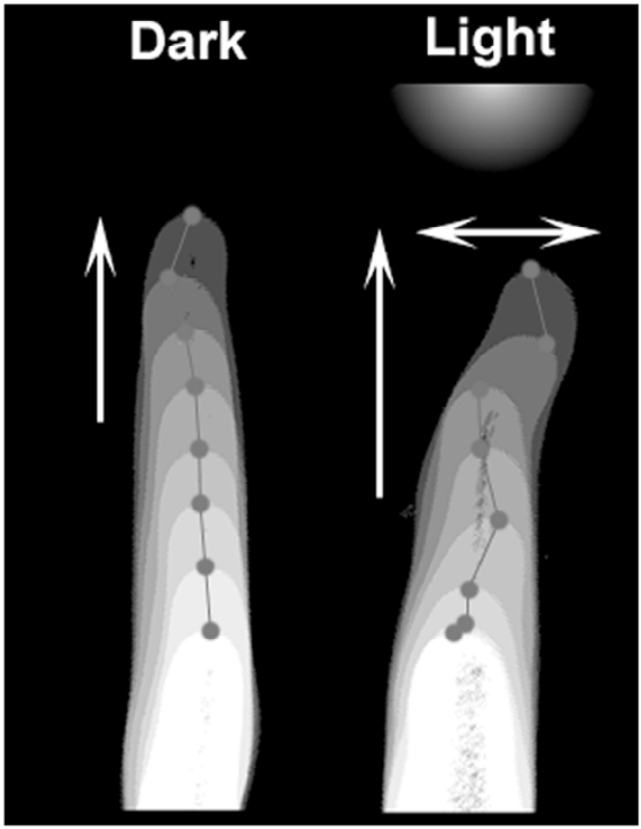

Figure 17. Light Induces the Lateral Serpentine Movement of Slug Tip.

Difference in the motion of slug tip in dark and during phototaxis. Consecutive eight images from slug migration sequences with time interval of 120 sec were binarized and averaged. The tip of the slug at each time point is marked with gray spot. The tip of the slug in dark progressed straightly, while that of phototactic slug oscillated laterally.

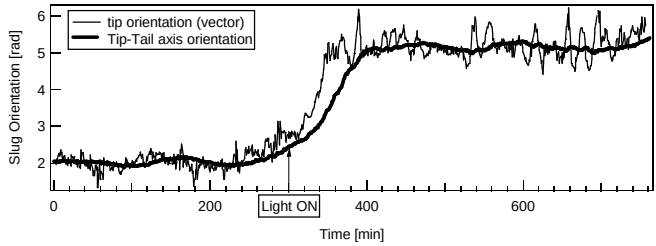

Figure 18. Tip Movement Vector and Slug Orientation During Phototaxis.

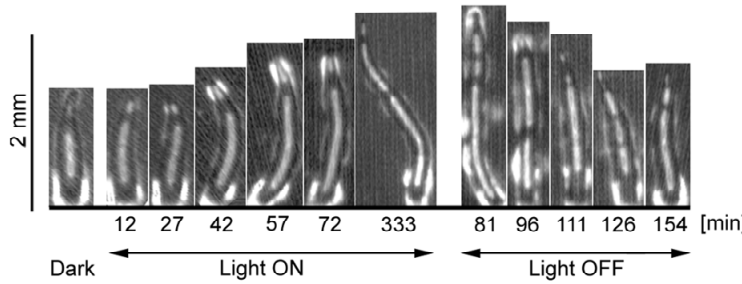

Figure 19. Process of Slug Length Change Following Turning On and Off the Light Source.

To study the process of length change in a different way, slug speed following light irradiation was analyzed at two different positions in the slug - the tip and the tail. **Figure 20a** shows that light irradiation increased the speed of the tip beginning after 10 minutes. By contrast, the increase in speed at the tail did not start until 60 minutes after light irradiation. **Figure 20b** shows the difference between tip speed and tail speed. As a result of the difference,

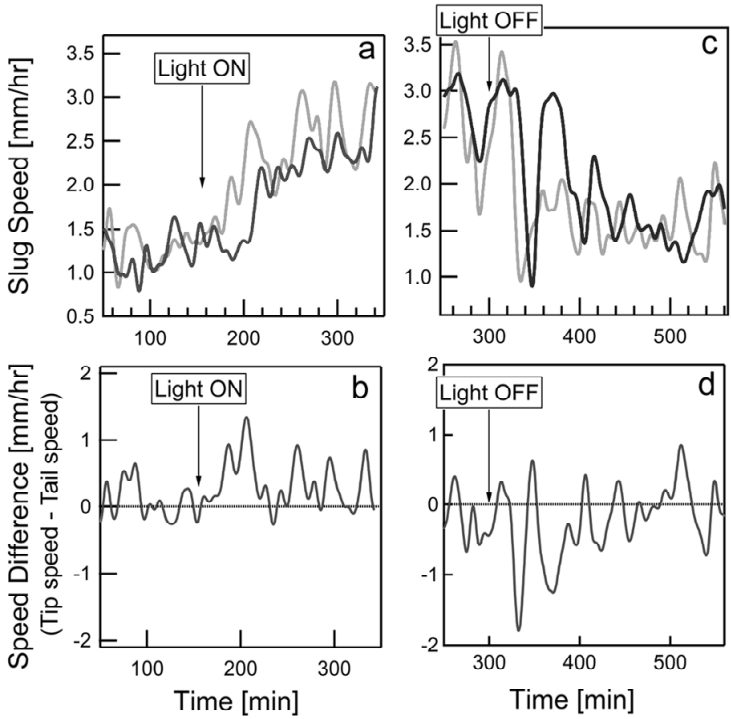

Figure 20. Delayed Speed Acceleration and Reduction of Tail Speed from the Tip Speed.

Unilateral light irradiation initiated and stopped at the indicated time points. (a). Increase of speed at tail (black) lagged behind that at the tip (gray). (b) Difference of tip and tail speed (Tip speed – Tail speed) obtained from the curves in (a). Positive increase of the speed difference after the light irradiation indicates the delay of the tip speed increase. (c) Decrease of speed at tail (black) also lagged behind that at the tip (gray). (d) Difference of tip and tail speed (Tip speed – Tail speed) obtained from the curves in (c). Negative increase of the speed difference after the light irradiation indicates the delay of the tip speed decrease.

the slugs lengthened. A similar asynchrony was observed after turning off the light source (**Fig. 20c,d**). The speed reduction at the tip occurred within 20 minutes whereas the speed reduction at the tail began after 70 minutes.

4.1.3 Prolonged lift off the substrate increases the tip speed

A possible mechanism for the speed increase following light irradiation could simply be that the rate of slug tip advancement is activated by light. This possibility was tested by measuring the acceleration of the tip that periodically occurred after the tip-landing on the agar (**Fig. 10, 12**). Since "blinking" indicated the tip-landing, speed change following every "blink" was determined and compared between dark migration and phototactic migration (**Fig. 21**). Unexpectedly, the acceleration of the tip in the dark and following light irradiation was similar. Linear fitting of the initial three minutes resulted in an acceleration of 9.78 mm/hr^2 in dark and 12.2 mm/hr^2 during phototaxis. Such a small difference can not explain 50% higher slug speed during phototaxis (**Fig. 12, 13**). By comparison, during the initial three minutes when slugs in dark and light condition were both increasing their speed, this difference in acceleration would result in speed difference of only 0.12 mm/hr, being only <10% of the average slug speed. On the other hand, **Fig. 21** shows that the duration of the acceleration was significantly longer during phototaxis. Approximate half maximum time was 1.7 min in the dark, compared to 2.6 minutes during phototaxis. Therefore, although the acceleration of slug tip was not significantly activated, phototactic slugs achieved higher speeds as a result of a prolonged acceleration phase. Another factor that caused the higher speed during phototaxis was a higher speed of phototactic slugs at time 0. Slug tips had an average speed of 0.88 mm/hr during tip-landing in the dark, compared to 1.25 mm/hr during phototaxis.

Previous work has documented a correlation between slug length and

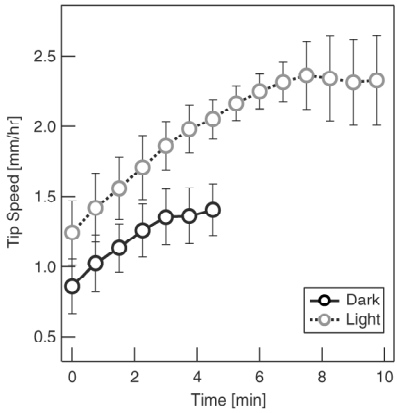

Figure 21. Kinetics of slug speed during dark migration and during phototaxis migration.

Acceleration phase of the periodic speed change of slug migration (see **Fig.10**, **12**, and **13**) were collected and averaged. Start point of the peaks were determined as the lowest speed point of the corresponding peak. Total of 6 slugs (68 peaks) migrating in the dark and 4 slugs (30 peaks) during phototaxis were averaged. The acceleration (*i.e.* slope) was similar regardless of light stimulation. On the other hand, the duration of increasing phase is much longer with phototaxis migration. Error bar = SD.

speed; longer slugs move faster (Bonner, 1995; Inouye and Takeuchi, 1979). The above measurements suggested that this correlation could occur in single slugs during a dynamic change of length and speed. By plotting a single slug's length against its speed, it became clear that this correlation holds also in an individual slug changing its speed during phototaxis (**Fig. 22**). Linear regression of this plot resulted a curve (black line) with a equation V=0.772 x L + 0.476, where V is the slug speed in mm/hr and L is the slug length in mm. This curve was similar to the regression curve derived from

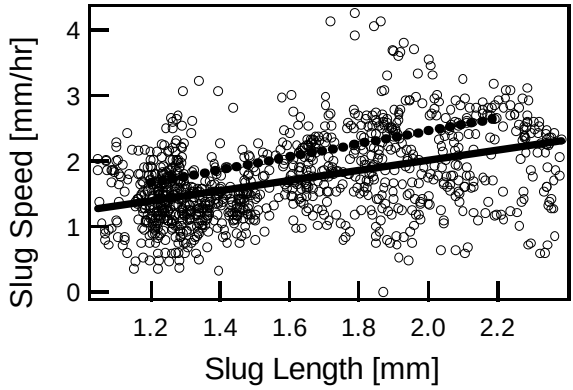

Figure 22. Dependence of Slug Length and Speed.

Slug speed showed strong dependence on the slug length. Slug speed was plotted against slug length at the corresponding time points. All points were derived from single slug (black line). Measurement from many individual slug was also plotted (dotted line; Bonner, 1995).

the measurement of many individual slugs (dotted line; V=0.85 x L + 0.65; Bonner, 1995).

4.1.4 Effect of ammonia

It has been reported that gaseous ammonia (NH_3) is given off by cells after light irradiation and that low partial pressures of NH_3 speed up slug movement while excess NH_3 impairs slug phototaxis (Bonner et al., 1988; Bonner et al., 1989). Based on these results, it was proposed that ammonia stimulates the migration speed of cells on the distal side of a slug thus turning the slug toward light (The differential speed hypothesis: Bonner et al., 1986).

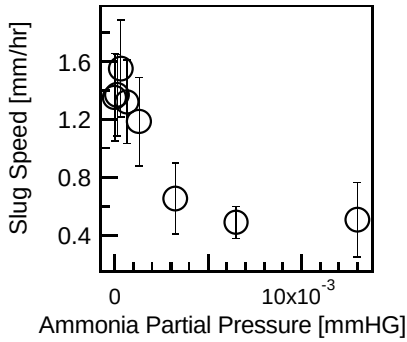

Figure 23. Effect of Ammonia on the Slug Speed.

Ammonia suppressed the slug migration speed in dose-dependent manner. More than ten slugs were measured for each of the ammonia partial pressure, from a couple of repeated experiment. Error bars = SD.

To test if the speed increase upon light irradiation is comparable to that following ammonia treatment, the speed of slugs under various partial pressures of ammonia was measured. Bonner *et al*. (1988) noted that single slugs transferred to fresh agar should be measured to avoid interference by ammonia accumulated during incubation before the experiment. Nevertheless, speeding up of slugs by light irradiation was observed while many other slugs were present on the same agar plate. To test in this condition, about ten slugs migrating on a single agar plate were treated with ammonia. Contrary to these earlier results, the migration speed decreased with increasing partial pressure of ammonia in the atmosphere (**Fig. 23**). The speed reached a minimum at a partial pressure of ammonia of 0.0065 mmHg. Bonner *et. al*. (1988) has documented a 11% increase of slug speed at a partial pressure of ammonia 0.0065 mmHg. The dose-response curve shown in **Figure 23** includes this partial pressure.

4.1.5 Effect of flickering light source

Since slugs have a tendency to maintain their direction of migration (Fisher et al., 1983), the directed migration of a slug toward a light source is also expected to be kept for a certain period of time after the light is turned off. There are two possible ways for the directed movement to decay after the light is turned off. The decay could be linear such that the directed movement decays constantly with time. In contrast, the slug may have a “short-term memory” of light direction after the light is turned off. The first case would suggest that the decay is analogous to the random migration in the dark in which spontaneous turning occurs by chance (Fisher et al., 1983). To test these possibilities, a light source was periodically turned on and off with various intervals and its effect on slug migration was tested. Slugs were allowed to migrate for 24 hours while they were irradiated by a unilateral flickering light source. The duration of on and off of light was kept equal so that the total time of darkness and irradiation was constant regardless of the interval length.

To assess the effect of flickering light on slug migration, slug trails were analyzed. Up to flickering interval of 20 min (20 min dark and 20 min light) slug trails were oriented more or less straight toward the light source indicating that the 20 min dark period did not affect the directed movement of the slug (**Fig. 24;** 5 to 20 min interval). For dark periods longer than 25 min there was a pronounced decrease in phototactic orientation. The trails became more winding and the straight approach towards the light source was lost (**Fig. 24;** 25 to 60 min interval). To study this effect quantitatively, the efficiency of phototaxis (EP) was calculated as the ratio of the total distance traveled to the net distance from the starting point (**Fig 25**). For example, EP is 1.0 if a slug migrated straight toward the light source. The EP decreased slightly from 5 min to 20 min of darkness. However, it dropped

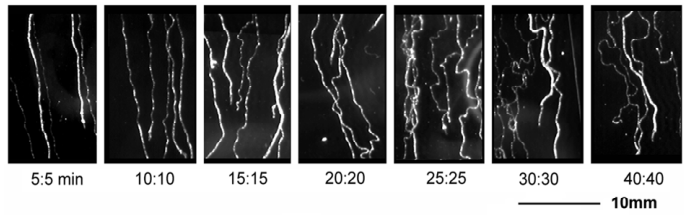

Figure 24. Effect of Dark Period on the Slug Orientation during Phototaxis.

A gallery of slug trails traveled when light was turned on and off with various intervals. White traces are slug trails. Orientation of slugs declined linearly from 5 minutes (indicated as 5:5 minutes) to 20 minutes of flickering interval. The trails of the slugs exposed to the flickering interval from 25 minutes to 40 minutes exhibited more frequent ”deviations” from the direction of the light source. Bar = 10 mm.

abruptly between 20 min and 25 min. EP remained at this level up to 60 min dark periods. Thus a critical change occurred between 20 minutes and 25 minutes, supporting the conjecture that the slug has a “short-term memory”.

4.2 Effect of light on slug cell movement

Irradiation with unilateral light not only induced phototactic turning but also elongation, thinning, speeding up and prominent serpentine movement of the tip. These dynamic changes in slug behavior and shape suggest that the organization of cell movement was altered within a phototactic slug. To study changes in cell movement within slugs in the dark, during phototactic turning and during persistent phototactic migration, cells labeled with a fluorescent dye were tracked and correlated with the corresponding slug

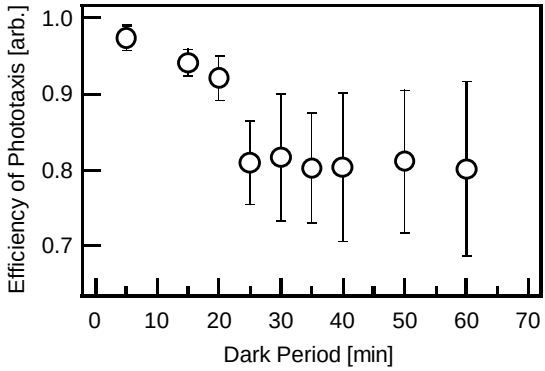

Figure 25. Period of Darkness and Efficiency of Phototaxis

The ratio of the net traveled distance towards light source to the total length of the trail was defined as the efficiency of phototaxis (EP). EP was plotted against the interval of the light source flickering. Each point is a mean value of the measurement of over ten slug trails from two similar experiments. Error bars are standard deviations.

movement. This method presented technical problems of focusing on the same cell moving in three dimensions and on the slug tip moving up-down. To overcome these difficulties, a second method was also used. Slugs with labeled cells were flattened under an agar sheet during phototactic stimulation. Cell movement could not be directly correlated with slug behavior in the second method since flattened slugs did not migrate but rotated at one place. However, it allowed more precise cell tracking for longer periods of time. Thus the effect of light on slug cells could be analyzed in more detail. Together with cell tracking results on un-flattened three dimensional slugs, it was possible to analyze cell movement during phototaxis.

4.2.1 Cell movement during phototactic turning of the slug

The bending of the slug anterior zone during phototactic turning is expected to be accompanied by a change in the relative positioning of cells within the slug. To test this, the the movement of cells labeled with the fluorescent cell marker DiR was analyzed within slug anterior zone. The wavelengths of excitation and emission of DiR are well above the action spectrum of slug phototaxis. Therefore cell movement could be observed both in the dark and during phototaxis without interfering with slug behavior. The analysis was done in two different ways. First, to detect the change in relative positioning of cells during turning, cell positions were connected by lines (**Fig. 26**). In this way, the change in relative positions could be detected as a change in the geometry of the "wire frame". Second, cell velocities were measured and the relationship of cell velocity and the turning of the anterior zone was analyzed (**Fig. 27**). Cell movement in the anterior zone could only be analyzed when the anterior zone was lifted above the agar substrate since under these conditions, the Z-position of the anterior zone was relatively constant and the cells remained in focus.

The slug shown in **Fig.26 a-d** was migrating straight forward in the dark. Cell movement was also essentially straight forward and the relative position of the cells did not change, as can be seen from the unaltered geometry of the wire frame (**Fig. 26 a-d**, gray lines). The cell velocity varied from one cell to the other, but there was no significant lateral movement (**Fig. 27 a-c**). By contrast, there was a clear difference in cell speed along the long axis of the slug. Cells closer to the anterior tip had higher velocities than those away from the tip (**Fig. 27 a-c**). Such a difference in the cell velocity widened the distance between the foremost cell and the last cell (compare black arrows in **Fig. 26 a** and **d**). These characteristics during dark migration were also observed in other experiments (n=3).

When light was irradiated, the slug anterior zone started bending toward

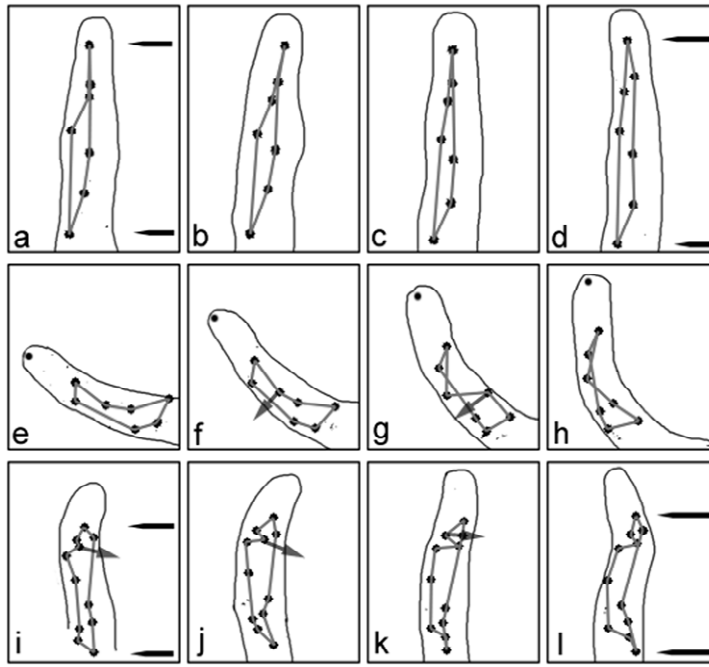

Figure 26 Wire frame diagram of anterior cell movement.

The anterior zone of a slug migrating in the dark (a-d), during the first large turning toward the light (e-h) and during oriented migration toward the light source (i-l) are each shown as four consecutive frames. Time between the frames is two minutes. Slug contour is shown in black line and positions of cells are indicated by black dots. Gray lines were drawn between the cells to show the geometry of cell positioning, so that the change in the relative position of the cells could be detected. (a-d) Slug was migrating toward the top of the frames. Slug tip proceeded straight forward in the dark with a slight lateral swinging of the tip. (e-h) Slug was migrating from right to left and then turned toward the top of the frame, in the direction of light source. (i-l) Slug was migrating toward the light source at the top of the frames. Anterior tip showed serpentine movement (**Fig. 17, 18**). Gray arrows in f, g, i and j show the direction of cell movement which is ”asymmetrically accumulating” during anterior zone bending (see Discussion). Black horizontal arrows in a, d, i and l point to cells at two positions along axis of the slug. An increase in the distance between those cells was detected.

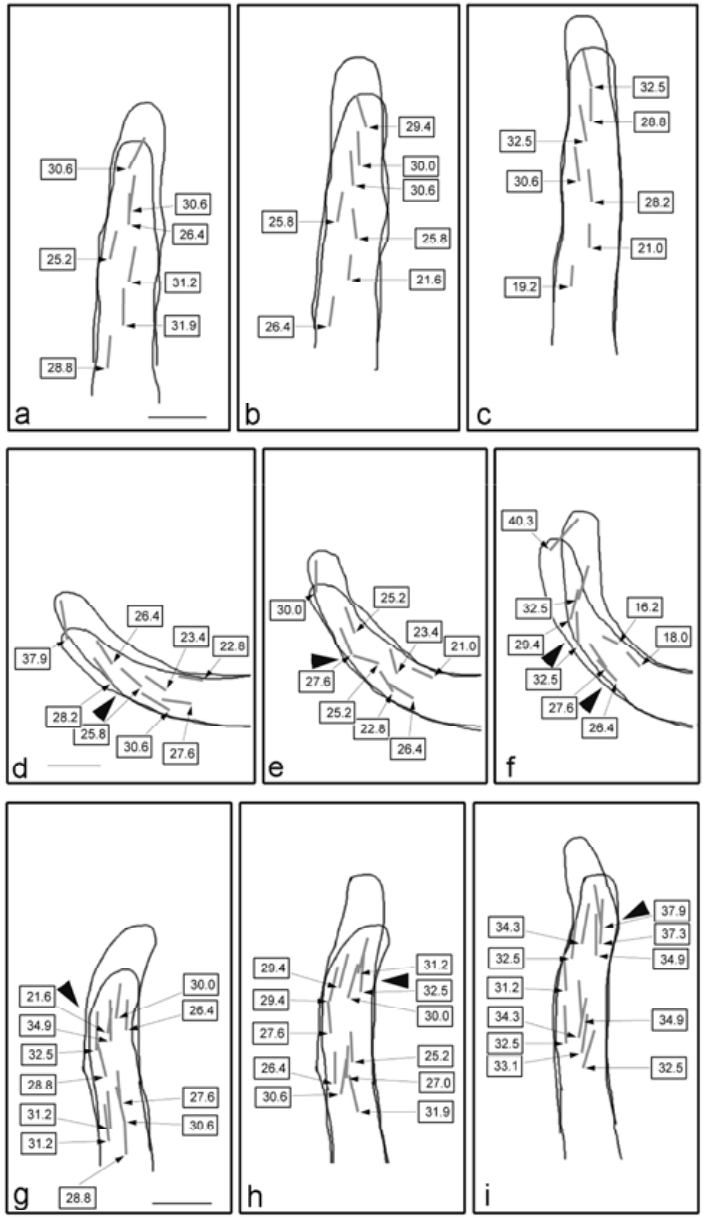

Figure 27. Anterior cell velocity distribution during phototaxis.

the light source. The bending of the anterior zone was like a lever-arm rather than a formation of new tip in the direction of light source. A clear change in the relative position of the cells was observed. Some cells which were on the irradiated side moved laterally towards the other side of the slug (n=5; **Fig. 25 e-h**, see gray arrows). At the same time a large difference in cell velocity between two sides of the slug could be detected. Cells migrating on the outside of the bending anterior zone moved at much faster speeds than those at the inside (**Fig. 26 d-f**). These differences corresponded well to the bending region (n=6; **Fig. 27 d-f**, arrow heads). It should be noted that the measured cell velocity contains two components; an active, self-propelled cell movement and a passive cell movement pushed by other cells. In the latter case, since the anterior zone is lifted off the agar, cells are pushed mechanically and increase in velocity by the movement of cells in the posterior part. For example, a cell at the front in **Figure 27f** moved at exceptionally high speed (40.3 µm/min). This movement is mostly passive and caused by the lever-arm like action of the bending, since position of the cell relative to the anterior zone did not change. Thus, self-propelled cell movement that bends the anterior zone should only be considered in the proximity of the lever-arm action (**Fig. 27d-f**, arrow heads).

Figure 27. Anterior cell velocity distribution during phototaxis.

The sequence shown in **Figure 26** was analyzed for the cell velocity. The contour of slug tip at a time point was overlaid with that after two minutes (black lines). Displacement of the DiR labeled cells during two minutes are shown as gray straight lines and the associated boxes indicate their cell velocity (µm/min) during that period. In this way, cell movement within slug and slug bending could be correlated. Arrow heads indicates the bending point. (a-c) Within the slug, cell tracks are mostly straight along the direction of slug movement. (d-f) Difference in the cell velocity between those cells on the outside and inside of the bend was detected (g-i) Anterior tip showed serpentine movement (**Fig. 17**) and cells at the bending point exhibit a difference in cell velocity between those at the outside and inside of the bend. Bar=0.1mm.

After the slug became oriented toward the light source, the tip of the slug showed serpentine movement due to a repetitive small bending of the tip (**Fig. 17**). Lateral change in the position of the cells was also detected in this case. **Figure 26 i-l** shows one of those repetitive turnings when the anterior zone was bending from right to left. At this instant, some cells moved from left to right (n=3; **Fig. 26 i-k,** see gray arrows). Such cell movement, a movement to the opposite side of the bending direction, was repeated during serpentine motion of the tip. Note that this is similar to cell behavior during initial turning (**Fig. 26 e-h**). The difference in cell speeds between the two sides alternated with repetitive bending. Cells on the outside of the bend moved faster than those on the inside (n=4; **Fig. 27 g-i**, see arrow heads). Although the cell velocity difference along the long axis is not as clear as it was during migration in the dark, there was indeed a widening of the distance between the foremost cell and the last cell (compare black arrows in **Fig. 26 i** and **l**)

To summarize, cells within the anterior zone during tip bending showed a characteristic change in their relative positioning. Some cells on the irradiated side moved to the unirradiated side of the slug. At the same time, cells on the unirradiated side moved faster than those on the irradiated side. This characteristic behavior was also observed during serpentine motion of the slug when the tip alternated its irradiated side from one side to the other.

4.2.2 Light effects on cell movement in flattened slug

Direct observation of light effects on cell movement as described in the previous section has several technical difficulties. In migrating slugs, target

cells tend to move out of the field of observation before changes in cell movement can be detected. In addition, cells moved out of the focus plane because of their three dimensional movement. Continuous focusing is more difficult when prestalk cells were observed because of an additional up-down motion of the tip. For these reasons, an alternative procedure was used in which slugs were overlaid with a thin agar film in order to flatten them slightly (Nicol et al., 1999). This restricted cell movement to two dimensions. Under these conditions, slugs became disc-shaped after one to two hours and showed strong rotational movement that continued for several hours. In some cases even a fruiting body formed, thus demonstrating the viability of the cells. Detailed analyses of cell movement were possible under these conditions since slugs rotated rather than migrating away and cell movement was confined within a flat space. To follow cell movement, prestalk cells were stained with the vital dye neutral red (Weijer et al., 1987). Upon light irradiation disc-shaped slugs showed several pronounced responses: first, neutral red stained prestalk cells speeded up, second, a group of the prestalk cells, the presumptive tip, migrated towards the periphery in a coordinated manner and third, the whole structure underwent repeated pronounced contractions (data not shown).

Although changes in cell velocity following light irradiation could be clearly observed in time lapse video sequences of neutral red stained slugs, the results could not be shown in a static image. The resolution was not sufficient for a precise measurement of cell speed. Furthermore, observation using near infrared light blurred the images and hindered single cell tracking. In order to analyze cell movement in slugs more precisely, 2~5 % of the slug cells were labeled with the cell marker DiR (**Fig. 28a,b**) and single labeled cells were tracked in slugs interactively. When flattened slugs were kept in the dark, cells rotated in the same direction at a constant speed. A sequence was processed to visualize the cell tracks (**Fig. 29**). Each frame of the sequence contained labeled cells such as shown in **Figure 28b**. Successive frames were overlaid so that the cell tracks could be visualized in a single

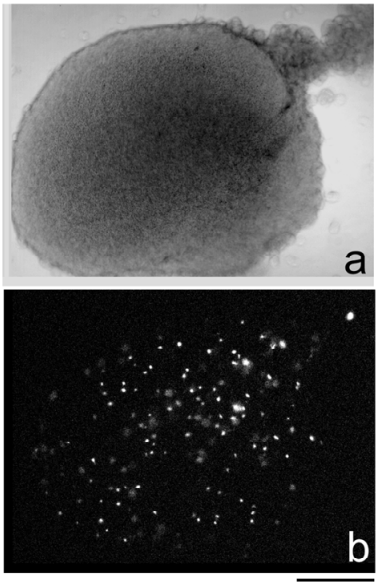

Figure 28. Agar overlay of DiR labeled slug.

(a) A phase contrast image of a slug 3 hours after the agar-overlay. (b) The same slug observed with epi-fluorescence microscopy. Approximately 2~5% of cells were stained with DiR. Bar = 0.1 mm.

frame. White lines and dots show cell tracks. When cell movement was slow, the track appeared as a line since cell displacement between frames was small. When cell velocity was high, the track became a dotted line since cell positions at different time points did not overlap. Cells at the periphery moved much faster (tracks in dots) than those closer to the center of the two-dimensional slug (tracks in lines), *i.e.* the cells seems to have similar angular speed.

The use of DiR instead of neutral red does not permit identification of prestalk cells but cell movement could be clearly followed. **Fig. 30a** shows that the average velocity of cells in the dark was 2~3 µm/min. The response of the DiR stained slugs to light was comparable to that of neutral red stained slugs: there was a visible change in speed of some cells and the slug underwent pronounced contractions (see above). The results of tracking individual DiR labeled cells indicated a significant increase in cell speed in about 15% of labeled cells (**Fig. 30b**). Since this proportion corresponds to the known

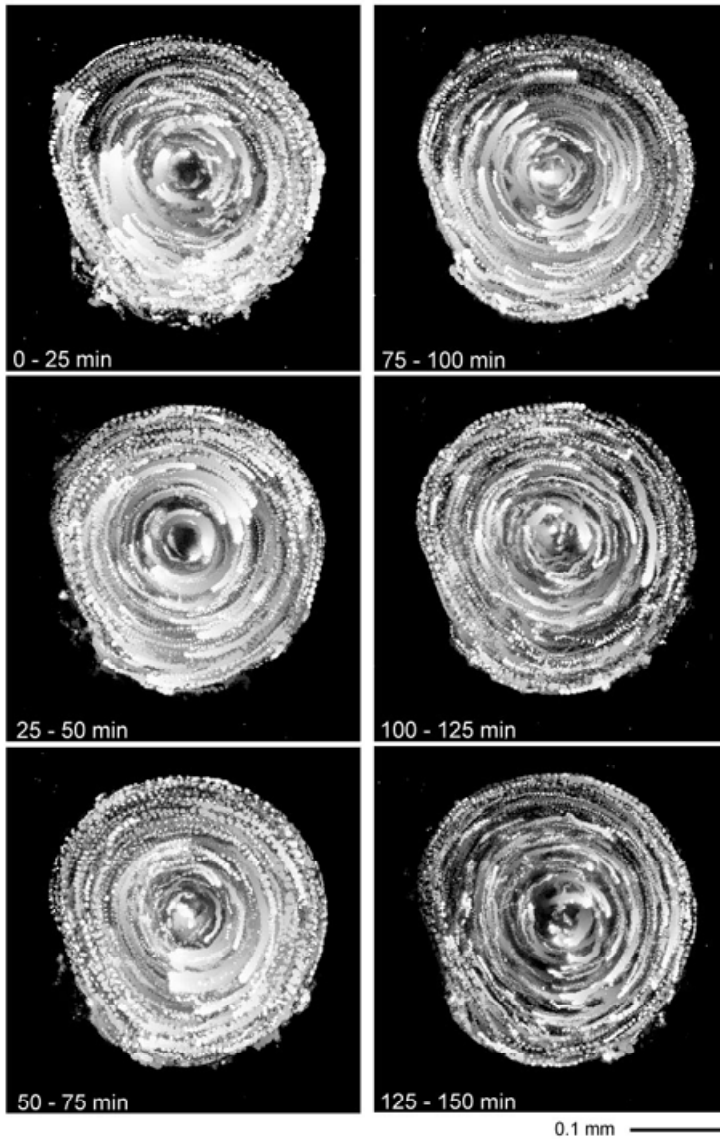

Figure 29. Rotational movement of the cells in flattened slug.

Flattened slug was kept in the dark. When cells were moving slowly, tracks were continuous. When cells moved faster, tracks became dotty.

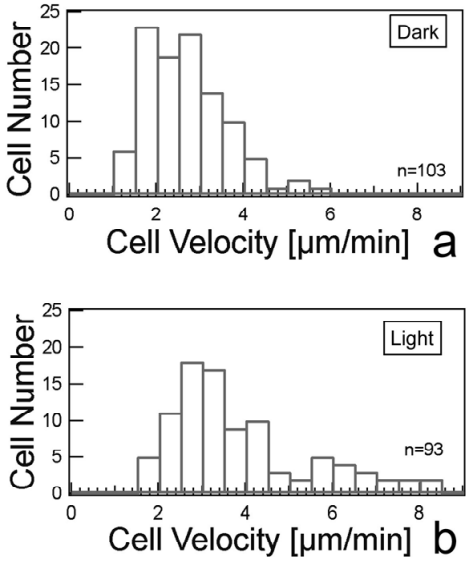

Figure 30. Slug cell velocity before and after the uniform light irradiation of disk-shaped slug.

The change in cell velocity before and after light irradiation was measured in slugs with DiR stained cells. (a) Distribution of cell velocities in dark (n=7) and (b) after light irradiation (n=6). The velocity was measured only in cells which could be tracked for the whole sequence before and after light irradiation (180 min.).

proportion of prestalk cells within slugs and since the response of these cells was similar to neutral red stained cells, it seems likely that this subpopulation corresponds to the prestalk cell population (Loomis, 1982; Sternfeld and David, 1982). There was also an increase in velocity of the remaining cells as shown in **Fig. 30b**. This increase was delayed relative to the response of the most active prestalk cells, suggesting that the light stimulus was first perceived by the prestalk cells and then transmitted to the prespore cells.

When a three-dimensional slug is irradiated unilaterally, the light focuses on the side of the slug distal to the light source. This lateral difference in

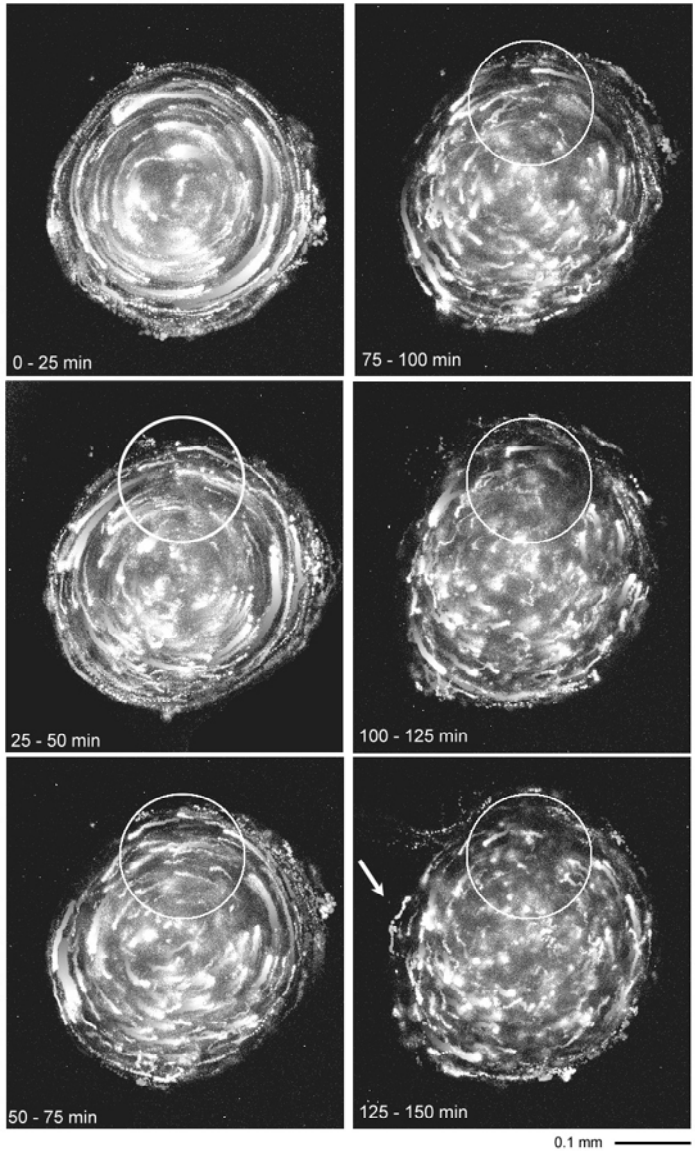

Figure 31. Effect of spot irradiation on flattened slug.

Light irradiation was started at 25 min. White circle indicates the irradiated area.

light intensity is thought to evoke the phototactic reaction. To mimic this condition, approximately one quarter of a flattened slug was irradiated with a light spot while the rest was kept dark. In **Figure 31**, a flattened slug was kept in the dark for 25 minutes; then light irradiation (white circle) was started at 25 minutes. The first reaction to such a partial irradiation occurred in the peripheral cells. They migrated towards the irradiated spot (dotty tracks on right side of the slug; **Fig. 31 25-50min**). Then some of them slowed down within the irradiated spot (**Fig. 31 50-75min**). The second reaction that occurred much later involved all other cells (**Fig. 31 100-150min**). They lost their regular circular movement, decreased their speed and their movement became disoriented. Then some of the cells clearly moved towards the light spot through the other cells (white arrows). In 6 out of 9 experiments, similar responses were detected. In two cases there were no responses and in one case all the cells speeded up.

4.3 Effect of light on cell-cell signaling

As described in the Introduction, many reports suggest that the multicellular movement of slugs is organized by cAMP signaling. Since light affects slug behavior and induces slug phototaxis, it appeared possible that light affects cAMP signaling directly. The following experiments were designed to test this idea. Light effects on cAMP signaling were demonstrated in aggregation cells, in streams and in slug tips.

4.3.1 Effect of light during aggregation stage

To test the effect of light on cell-cell signaling, cells in aggregation phase were irradiated with light and their activity was analyzed. Two hours after starvation individual amoebae migrated randomly (**Fig. 32a**). At this time point cell movement activity, assayed as net movement between two images

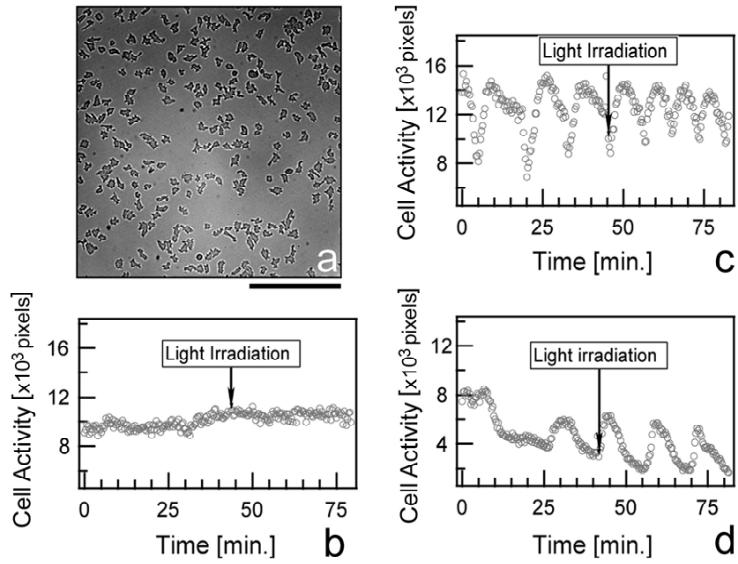

Figure 32. Light Increases cAMP Oscillation Frequency during Early Aggregation.

Cells in aggregation phase were analyzed for their change in movement activity upon light irradiation. Cells migrating on an agar substrate in a dark chamber (a) were starved in the dark for 2 hrs (b), 4 hrs (c) and 6.5 hrs (d). Images were taken with infrared light. Irradiation with active light started after recording approximately 40 minutes in the dark. Cell activity is a measure of the change in cell shape between two consecutive image frames (in pixels x 10^{-6}) for the whole field of cells. Bar = 0.2 mm.

of a time lapse series, was constant in dark conditions (**Fig. 32b**; see materials and methods for details). Even though some cells may move periodically (Wessels et al., 1994), the periodicity is averaged out by summing the activity of a few hundred cells. Light irradiation of the whole observation field did not change this constant level of activity (**Fig. 32b**). By four hours after starvation the amoebae began to show periodic changes in cell movement activity indicating that they were in aggregation phase (**Fig. 32c**) and that their movement was being synchronized by cAMP waves

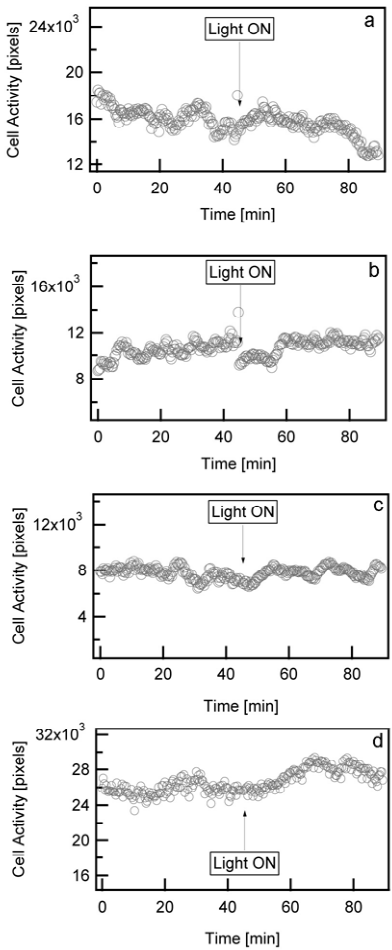

Figure 33. Light did not Affect the Cell movement Activity of Chemotaxis Deficient Mutant.

Same experiment as shown in **Fig. 32** was repeated with mutant strain KI8, which do not aggregate since the mutant lacks chemotactic ability. Cells migrating on an agar substrate in a dark chamber were starved in the dark for 2 hrs (a), 4 hrs (b), 6.5 hrs (c) and 24 hrs (d). Cell activity did not change after light irradiation.

which propagated across the field of observation. The amoebae increased their movement activity when cAMP increased and transiently decreased their movement activity when they adapted to the stimulus and the cAMP concentration decreased again (Alcantara and Monk, 1974; Siegert and Weijer, 1989; Tomchik and Devreotes, 1981). In the dark, five oscillations per hour were observed which corresponds to an average period length of 12 minutes (**Fig. 32c**). After light irradiation the period of oscillation

decreased quickly to an average of 8.5 minutes (**Fig. 32c**). This effect was even more pronounced at six hours after starvation (**Fig. 32d**). When cells were kept in the dark without light stimulation for a similar period of time, no such change in the frequency occurred.

To confirm that the light effect was solely due to the increase in cAMP signaling frequency and not to an increase of cell activity, a mutant strain was tested. Mutant strain KI8 lacks the chemoattractant stimulated intracellular cGMP transient which triggers chemotactic movement. Thus, KI8 cells do not chemotax and aggregate (Kuwayama et al., 1993). Since KI8 cells are defective in chemotactic movement the effect of light on the cell activity could be tested directly. The KI8 cells were irradiated at 2, 4, 6.5 and 24 hrs after the starvation. Measurements of cell activity showed that light irradiation did not change the level of cell activity at any stage (**Fig. 33 a-d**). Cell movement was unsynchronized and randomly directed both in the dark and after light irradiation.

4.3.2 Light induced new waves in streaming stage

Light also induced changes in cAMP signaling at later stages when cells formed cell-cell contacts and were migrating in aggregation streams. In the aggregation stream shown in **Fig. 34a**, the aggregation center was outside the image to the right. Cells were thus moving from left to right, while cAMP waves could be detected under the dark-field illumination as waves of periodic cell shape change propagating from right to left. **Fig. 34b** shows a time-space plot (Siegert and Weijer, 1989) in which the dark-field waves are shown as tilted dark lines. X-axis corresponds to the length of the stream in **Fig. 34a**. The intensity of vertical one pixel wide columns in the white square was averaged for each column along the length of the stream and then the average values were represented as single pixels in **Fig. 34b**. This procedure resulted in a horizontal bar with one-pixel height and the width

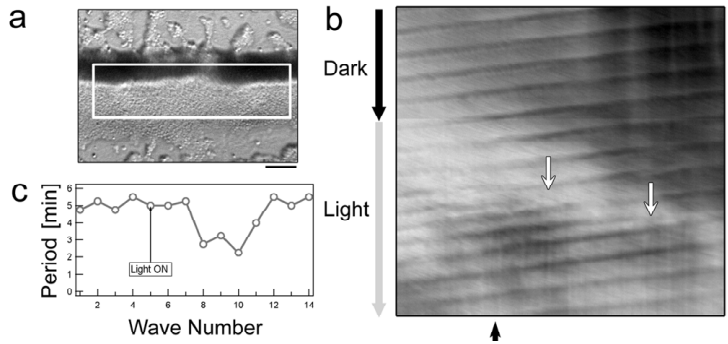

Figure 34. Light Induces the Formation of New cAMP Waves in Aggregation Streams.

(a) A bright-field image of an aggregation stream stretched horizontally. Waves were hard to detect by eyes from the static image. It could be only detected as a slightly dark vertical bands in this image. The white frame indicates the window which was used for time-space plot acquisition. (b) Time space plot showing dark-field waves propagating from right to left along the stream. Periods were measured at the position indicated by the black arrow at the bottom of the figure and were plotted in the graph (c). Bar = 0.2 mm**.**

of the white window. The procedure was repeated for every frame of the sequence and the bars were aligned from top to bottom along y-axis to show the temporal change in the position of dark field wave along the length of the stream. Thus, tilted dark lines in the figure show the change in the position of dark-field waves within the stream. Images were taken every 20 seconds. Light irradiation was started at the time indicated on the ordinate and was confined to the region under the illumination field of the microscope. A transient increase in the frequency of dark-field waves is visible in the time-space plot as a narrowing of the spacing between successive waves. Since the stimulating light only illuminated a small spot of the stream, which did not include the aggregation center, this increase is due to new waves induced at the irradiated spot and not at the aggregation center (**Fig. 34b, white arrows**). When the stream was kept in the dark without light

stimulation for a similar period of time, no such induction of waves occurred. The propagation speed of the waves remained constant as indicated by the unaltered slope of the band pattern. The spacing between dark-field waves corresponds to the period of each wave. **Fig 34c** shows the period of each of the successive waves. A transient decrease in the period occurred within ten minutes after light irradiation. From these experiments it was concluded that light acts on the dynamics of cAMP relay during late aggregation.

4.3.3 Light induced cAMP emission from the slug tip

To assay light effects on cell-cell signaling in slugs, slug tips placed in a field of aggregation competent cells were irradiated with light. Transplanted tips attract surrounding cells and act as aggregation centers by releasing cAMP (Bonner, 1949; Maeda, 1977; Rubin and Robertson, 1975). If light affects cAMP signaling in the tips, a change in chemotactic activity of the aggregation competent cells is expected. Tips were transplanted to a field of pre-aggregation cells which could respond to cAMP but not to light (see **Fig. 32b**.) In the dark, cells showed a weak tendency to migrate toward the transplanted slug tip due to diffusion of small amounts of cAMP (Maeda, 1977; Rubin and Robertson, 1975); **Fig. 35 a-c**). However, after ten minutes of light irradiation we observed a dramatic increase in chemotactic activity (**Fig. 35f**): most of the cells in the field migrated straight toward the transplanted tip. The velocity of cell movement was not affected by light.

There are several possible explanations other than chemotaxis, why cells surrounding the slug tip respond to the irradiated light with directed movement towards the slug tip. For example, the directed movement could be due to galvanotaxis or cells could simply increase their persistence of movement. To confirm that the light-induced cell behavior was chemotaxis, cells of the non-chemotactic mutant strain KI8 were used as the detector cells and the experiment was repeated (Kuwayama et al., 1993). Mutant

cells migrating in the vicinity of transplanted tip did not show oriented movement towards the tip either before or after light irradiation (**Fig. 36**). To further test whether the emitted chemoattractant was cAMP, the experiment was repeated with mutant cells lacking cAMP receptors. In this mutant strain, both the *car1* and *car3* genes encoding cAMP receptors are deleted. The cells are not chemotactic toward cAMP and are not able to aggregate (Insall et al., 1994). The *car1-/car3-* cells did not show oriented movement towards transplanted tips either before or after light irradiation (**Fig. 37**) indicating that cAMP is the chemotactic signal released by tips following light irradiation. The *car1-/car3-* cells had normal chemotactic activity when folic acid was used as a chemoattractant (**Fig. 38**).

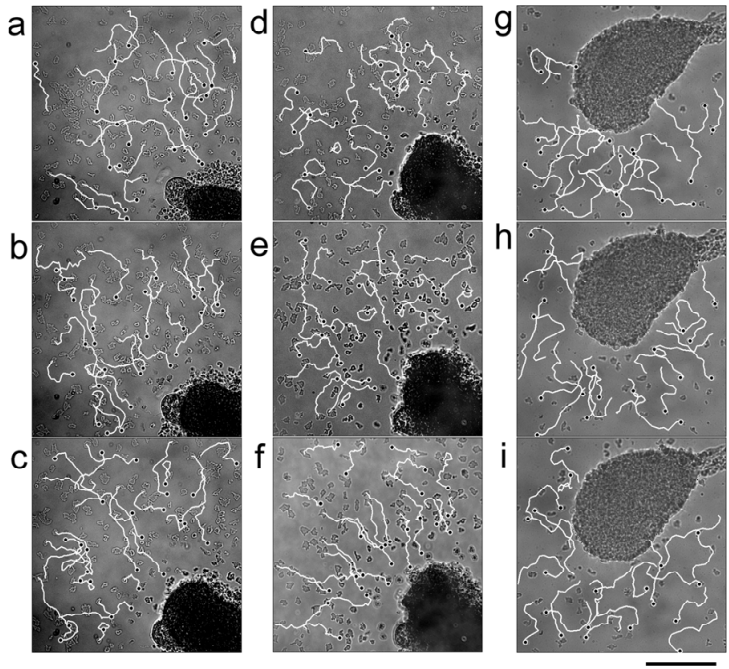

Figure 35. Slug Tips Release cAMP upon Light Irradiation

A dissected slug tip was placed in a field of pre-aggregation cells and overlaid with an agar film. Each column shows 3 consecutive 10 min periods from 50 to 80 minutes after the overlay. White lines indicate cell movement during the 10 min period; black dots indicate the final positions of the cells. (a-f) NC-4 cells in the field, showing 6 consecutive 10 min periods from 20 to 80 minutes after the overlay. The tip is visible at the right-bottom corner of each image. Light irradiation was started 60 minutes after the agar overlay (e and f). The experiment was repeated six times with the same result. The slug tip is visible at the center of each image. (g-i) NC-4 cells in the field, showing 3 consecutive 10 min periods from 50 to 80 minutes after the overlay in the dark. Bar = 0.2 mm.

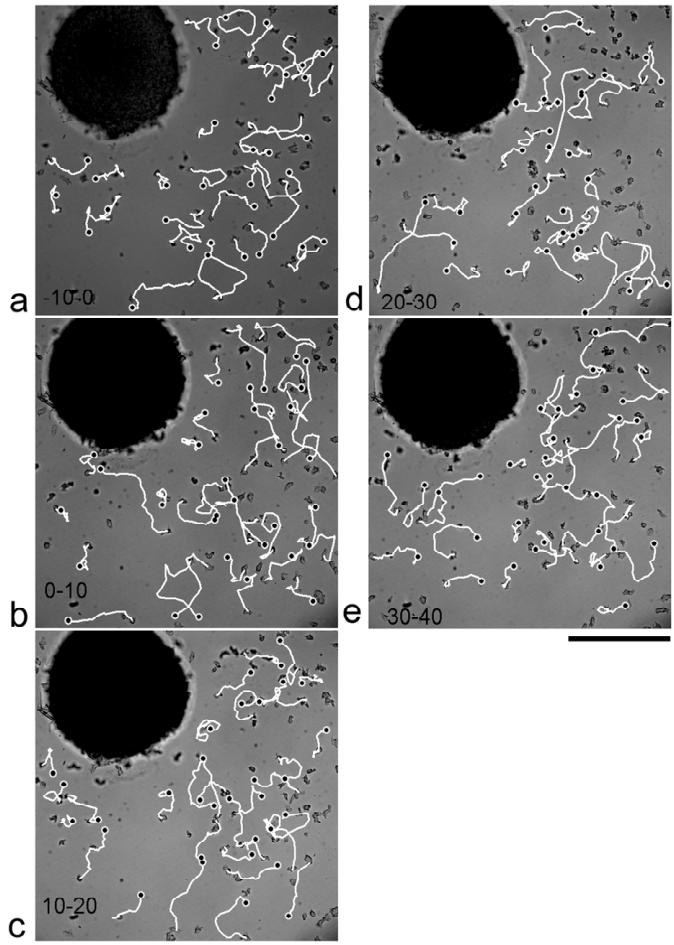

Figure 36. Mutant KI8 cells do not respond to the light irradiated slug tip.

Same experiment as **Fig. 35** was repeated, except that mutant KI8 cells were placed in the field. 5 consecutive 10 min periods from 50 to 100 minutes after the overlay are shown. White lines indicate cell movement during the 10 min period; black dots indicate the final positions of the cells. Light irradiation was started 60 minutes after the agar overlay (b). Mutant cells showed no orientation toward the tip. Bar = 0.2 mm.

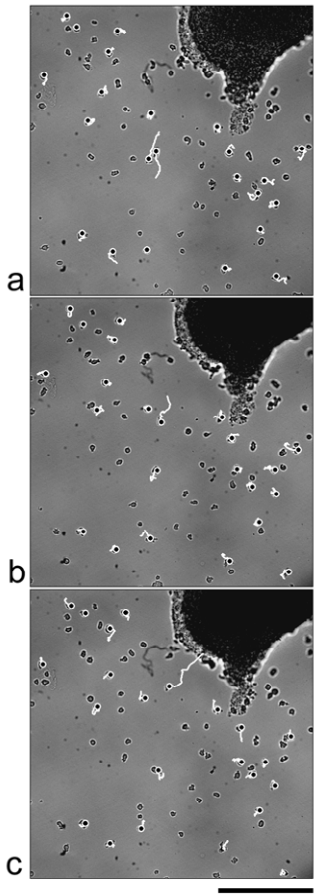

Figure 37. Mutant *car1-/car3-* Cells do not respond to the light irradiated slug tip.

Same experiment as shown in **Fig. 35** was repeated, except that mutant *car1-/car3-* cells were in the field. 3 consecutive 10 min periods from 50 to 80 minutes after the overlay are shown. White lines indicate cell movement during the 10 min period; black dots indicate the final positions of the cells. Light irradiation was started 60 minutes after the agar overlay (b). Mutant cells were not as motile as wild type cells (short tracks) and showed no orientation toward the tip. Bar = 0.2 mm.

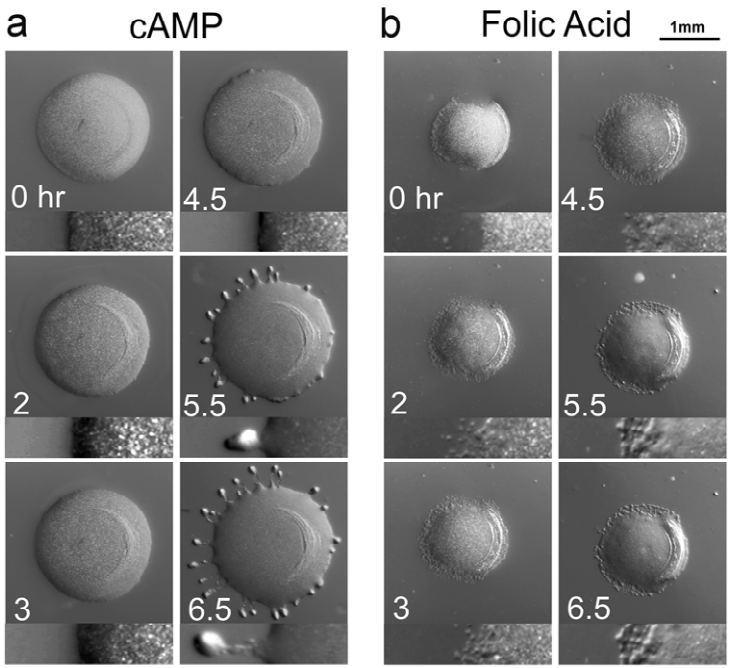

Figure 38. Chemotactic Behavior of car1-/car3- Cells.

Washed *car1-/car3*- cells (10^8 cells / ml) were spotted on the KK_2 agar containing (a) 100 µM cAMP or (b) 1 mM folic acid at 0 hr. The magnified view of the spot edge is shown at the bottom of each frame. Cells migrated out from the spot on folic acid by 2hrs but not on cAMP. Mutant cells on the cAMP plate did not show chemotactic behavior up to 4.5 hrs after they were placed on the plate. Cells on the cAMP plate started outward migration between 4.5 and 5.5, indicating expression of a cAMP receptor other than *car1* or *car3* by this time.

5 Discussion

The behavior of migratory multicellular slug stage of *D. discoideum* has been studied at two different levels; overall slug behavior (macroscopic) and cell movement (microscopic) within migrating slugs. Particular attention has been given to slug phototaxis and the results suggest that light stimulates the cell-cell signaling system by inducing cAMP release. Thus behavior at the microscopic level can be linked to behavior at the macroscopic level by cell-cell signaling and provides insights into how slug phototaxis is brought about.

5.1 Light-induced changes in slug behavior

Observations of migrating slugs in dark and light showed that slugs undergo several pronounced morphological and behavioral changes following light irradiation: 1.) the migration speed increased by 50%, 2.) the overall slug shape changed from short and fat to long and thin, 3.) tip movement changed from straight to serpentine-like motion; and 4.) the period of the up-down motion of the tip doubled in length. All these effects were completely reversible, when the light source was turned off. These results collectively suggest that light affects the coordination system of slug movement.

5.1.1 Slug speed increases following light irradiation

Whether a slug increases its speed during phototaxis has been of special interest in terms of testing two phototaxis hypotheses, the differential speed hypothesis (Bonner, 1994) and the tip activation / inhibition hypothesis (Fisher *et al.*, 1984). The former predicts a speed increase during phototaxis while the latter predicts unaltered speed. Previous tests of these predictions have yielded conflicting results. There are reports of an increase in slug

speed following light irradiation (Kitami, 1982; Poff and Loomis, 1973) and there are reports that slugs do not change speed upon light irradiation (Bonner and Whitfield, 1965; Raper, 1940; Smith *et al.* 1982). The results presented in this thesis document an increase in slug speed following light irradiation (**Fig. 12**). Furthermore reexamination of the earlier results of Smith *et al.* (1982) shows that light increased slug speed when the results were not normalized for slug length. Since it is now clear that slug length varies with slug speed during the phototactic response (**Fig. 15, 16´, 22**), it is clear that normalization of slug speed to slug length obscures speed changes following light irradiation (review; Fisher, 2000). These findings resolve the discrepancy in the previous results and indeed now show that these reports all document an increase in the slug speed following light irradiation.

5.1.2 A reduced frequency of up-down motion is responsible for the speed increase of the slugs

How can a slug speed up? It is unlikely that the tip increases its tractional force against the substrate since the tip is lifted off the agar for the most of time during migration (see also Inouye and Takeuchi; 1979)[1]. It is more likely that morphological change, *i.e.* elongation, is the mechanism of speeding up. Upon light irradiation, the diameter of the tip became progressively thinner over time (**Fig. 19**). As it becomes thinner, the anterior zone elongates since its volume is constant. Since one end of the anterior zone is lifted off the substrate while the other end rests on the relative immobile prespore zone, elongation causes advancement of the tip.

It was found that the slug tip periodically lands on and lifts off the agar

[1] These results are also in agreement with previous observations that cells in prestalk zone are not producing the driving force for forward movement of the slug by directly exerting traction against the substrate since they are moving tangentially to the long axis (Siegert and Weijer, 1992).

surface. This periodic movement is coupled with an increase and decrease in tip speed (**Fig. 10**). The acceleration of the speed is initiated by the lift off from the substrate (**Fig. 10, 12**; Breen and Williams, 1994; Inouye and Takeuchi, 1979). The analysis showed that this acceleration was unaffected by light irradiation (**Fig. 21**). In addition, the analysis showed an increase in the distance between the foremost cell and the last cell in the anterior zone (**Fig. 26 a, d** and **i, l**) regardless of the light condition. Considering that the substrate works as an obstacle to tip advancement (Bonner, 1995)[2], prolonged lifting of the tip during phototaxis enables prolonged elongation of the anterior zone which results in faster movement (**Fig. 21**). As a consequence the whole slug elongates although the speed increase at the posterior zone is delayed (**Fig. 20**). A reduced frequency of up-down motion is thus responsible for the observed speed increase as well as the elongated thin shape of phototactic slugs (**Fig. 12, 15** see also Bonner, 1995). This is in good agreement with an earlier report showing that faster slugs have sharper and thinner tips (Francis, 1962).

5.1.3 Phototactic slug turning by asymmetric cell accumulation

Several hypotheses have been proposed on how a turn towards the light is made. The differential speed hypothesis suggests that the movement of cells on the side of the slug distal to the light source is activated and that the difference in cell speed across the tip bends the anterior zone toward the light source (**Fig. 2b**; Bonner, 1994). By contrast, the tip activation / inhibition hypothesis suggests that light activates the formation of a new tip on the side proximal to the light source thus causing the extension of a new tip toward the light source (**Fig. 2c**; Fisher *et al.*, 1984) [3].

[2] When the slug tip was artificially held lifted off the agar surface by lateral static electric force, slug became longer. Breen and Williams (1994) also reported an abrupt slowdown of both the slug tip speed and slug cell speed upon the landing of the tip on the agar surface.

To test these hypotheses, cell movement within phototaxing slugs was analyzed in detail. Cell movement during turning, however, was found to be different from that predicted by both hypotheses. During a turn towards a light source, some cells moved across the tip toward the distal side (**Fig. 26, 27**). This movement increased the volume on the distal side while decreasing the volume on the irradiated side, causing a bending of the tip towards the light source like a lever arm (**Fig. 39**; Asymmetric cell accumulation hypothesis. See also **Fig. 41b**). Although some speeding

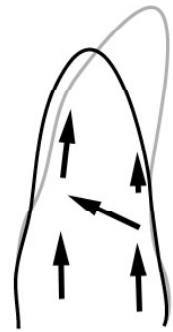

Figure 39. Asymmetric Cell Accumulation Hypothesis.

Black line: contour of the slug anterior zone. Gray line: The contour after the turning. Arrows show the observed cell movement vector.

[3] Tip activation/inhibition hypothesis was formulated based on an analogy to slug morphogenesis. Slug morphogenesis, which automatically generates a single tip without external signals, obviously needs a coupled autoactivation and autoinhibition process. On the contrary, in the case of phototaxis, tip formation is induced by an external cue *i.e.* unilateral light. The condition for the polarity generation is not the same as self-organization. It might be more suitable to re-formulate the hypothesis that light "modulates" the putative tip activation signal (*i.e.* not the tip activation *de novo*) and that the tip inhibition signal is not necessarily be included into the hypothesis to orient the tip towards the light.

up of cells on the distal side was observed (**Fig. 27**), this could be due to "passive" acceleration by volume expansion as a result of the asymmetric accumulation (**Fig. 39**). An extreme example of this was a cell at the very tip of the slug in **Figure 27f**. Measurements showed that the cell moved at 40.3 µm/min. Careful examination of the images, however, showed that this was due to the lever arm action of the bending tip.

5.1.4 Serpentine motion is phototaxis

When a slug completed turning and became oriented toward a light source, it showed small repetitive turns which were termed serpentine movement. During serpentine movement the tip swung laterally back and forth between both sides of the light axis as if it was scanning the position of light source (**Fig. 17, 18**). Asymmetric cell accumulation was detected during these small repetitive turns (**Fig. 26 i ~ l**), supporting the idea that the serpentine movement was the result of small phototactic turns. The net outcome is the oriented migration toward the light source.

In slugs, the frequency of serpentine movement was higher than the frequency of up-down motion: a single turn of serpentine motion (*e.g.* left → right) occurred every 5 ~ 10 min (**Fig. 18**) while period of up-down motion was approximately 30 min (**Fig. 12, 13**). Thus, a tip can bend several times laterally while the anterior zone is lifted off the substrate. Since serpentine movement is caused by phototaxis and can only occur when the tip is lifted off the agar, the efficiency of phototaxis is influenced by the frequency of up-down motion. Frequent up-down motion may thus decrease the ability of the slug tip to turn towards the light source. In fact, it is interesting to note that poor phototaxis has been associated with a decreased slug speed (*i.e.* higher frequency of up-down motion) in a number of experiments (**Table 1**).

Table 1. Phototaxis deficiencies and slug migration speed in previous reports.

Treatment or Mutation	Phototaxis orientation	Slug speed compared to the wild type	Reference
Ammonia 0.005mmHG	Less accurate	Similar	Bonner et al., 1988
Ammonia 0.008mmHG	Random	Slow (-50%)*	Bonner et al., 1988
Pertussis toxin 200ng/ml agar	Less accurate	Slow (-30%)*	Darcy & Fisher, 1989
KF 7.5 mM	Less accurate (bidirectional)	Slow (-40%)*	Dohrmann et al., 1984
Ca++ 2.5 mM	More accurate	Similar	Dohrmann et al., 1984
Ca++ 7.5 mM	Less accurate	Similar	Dohrmann et al., 1984
EGTA 3 mM	Less accurate (bidirectional)	Similar	Dohrmann et al., 1984
Rm- (regulatory subunit of PKA)	Less accurate	Slow (-64 %)	Bonner & Williams, 1994
Strain L-20, L-25 (mutation unknown)	Random	Slow	Loomis 1970
HU410	Less accurate (bidirectional)	Slow (-50%)*	Fisher, 1981
HPF229 (cGMP kinetics altered)	Random	Slow (-80%)*	Darcy et al., 1994
HPF230 (cGMP kinetics altered)	Less accurate (bidirectional)	Slow (-30%)*	Darcy et al., 1994
ABP120- (HG1270)	Less accurate (bidirectional)	Slow (-50%)*	Wallraff & Wallraff 1997 Fisher et al., 1997
GRP- (gelsolin-related protein)	Less accurate	Slow (-30%)*	Stocker et al.,1999

* Extent of speed decrease was estimated from the slug trails shown in the reference figures.

5.2 cAMP secretion and cell movement during phototactic turning

What causes the cells to accumulate asymmetrically during phototactic turning? And how does the slug decrease the frequency of up-down motion? Light effects on cAMP signaling suggest answers to these questions (**Fig. 32 ~ 38**). Experiments showed that light irradiation increased the frequency of cAMP pulsing both before and after the formation of cell-cell contacts at the aggregation stage. Light also stimulated the release of cAMP from slug tips. Taken together, these results indicate that light stimulates cAMP pulsing in the cell-cell signaling system. This is the first direct evidence of an influence of light on cAMP signaling and is in good agreement with previous reports that cAMP signaling is involved in phototaxis (Bonner and Williams, 1994; Bonner, 1994; Darcy and Fisher, 1990; Fisher, 1997; Fisher, 2000).

Previous work has shown that cell movement within the slug is organized by propagating waves of cAMP (Bretschneider et al., 1995; Durston and Vork, 1979; Rubin, 1976; Siegert and Weijer, 1992; Siegert and Weijer, 1995; Sternfeld and David, 1981; Traynor et al., 1992). These waves organize tangential rotation in the anterior zone and forward movement of cells parallel to the long axis in the posterior zone (Siegert and Weijer, 1992). When slugs are unilaterally irradiated with light, the geometry of the propagating cAMP wave would be asymmetrically modulated since light irradiation stimulates cAMP secretion by slug cells (**Fig. 35**). As other cells follow the altered geometry, the trajectories of the cells would also be modulated. Indeed, the analysis of cell movement within slugs showed that light irradiation modulates their movement pattern.

5.2.1 Release of cAMP activates cell movement and collects prestalk cells at the site of light irradiation

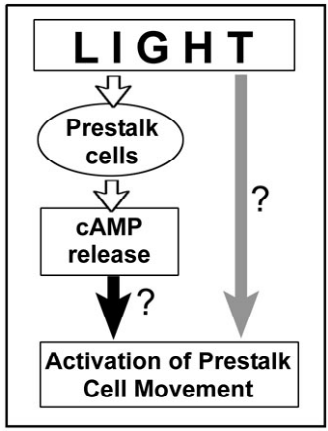

Figure 40.

A possible mechanism of light induced activation of prestalk cell movement.

It was found that light irradiation increased cell movement activity in flattened disc-shaped slugs (**Fig. 30b**). This could be due either to a direct effect (**Fig. 40**; gray arrow) or an indirect effect of light on cell movement (**Fig. 40**; black arrow). Although there is no direct evidence, it appears more likely that the light effect on cell movement is an indirect result of light induced stimulation of cAMP release. Several findings support this view. First, light stimulated aggregation stage and slug stage cells but did not activate the movement of pre-aggregation cells (**Fig. 32b**). Thus, the light response required the transition to the multicellular stage and was correlated with the development of cAMP signaling system. Second, light was shown to stimulate cAMP release from the slug tip cells (**Fig. 35**) and hence increased levels of cAMP are expected to occur in two-dimensional slugs following light irradiation. This predicted increase in cAMP is correlated with an increased movement activity of neutral red stained prestalk cells (see also **Fig 30b**) and thus is consistent with published results showing that prestalk cells exhibit a strong chemotactic response to cAMP (Early et al., 1995; Mee et al., 1986; Sternfeld and David, 1981; Traynor et al., 1992; Wang and Schaap, 1985).

Third, a similar response was also detected when flattened disc-shaped slugs were irradiated with a spot of light. In such a flattened slug, actively moving prestalk cells localized to the periphery (**Fig. 29**). The first effect of spot irradiation was to activate migration of these prestalk cells (**Fig. 31 25-75min**) toward the irradiated spot. Since the irradiated spot and the responding cells were spatially separated, the observed response could not be a direct effect of light but was more likely a result of cAMP secretion in the irradiated spot and subsequent chemotactic attraction.

Secondary reactions occurred after long exposure to the spot of light (**Fig. 31 75-150min**). Circular movement became distorted and some cells even showed movement opposite to the circular movement of other cells. The accumulation of released cAMP due to the continuous light irradiation probably interfered with the movement of these cells. Taken together, these results indicate that the light induced cAMP secretion can activate cell movement and collect prestalk cells to the site of light irradiation.

5.2.2 Asymmetric accumulation of prestalk cells

Since local irradiation of flattened slugs caused accumulation of active (*i.e.* prestalk) cells (**Fig. 31**), it seems possible that the emission of cAMP on the distal side of the slug where irradiated light is focused could cause asymmetric cell accumulation in intact (=non-flattened) slugs. Indeed cell movement patterns within turning slugs suggest that light induces formation of a secondary cAMP source on the distal side of the slug (**Fig. 26**). This secondary source did not interfere with the preexisting tip (the primary aggregation center).

Formation of such a secondary cAMP source has also been observed in other cases. When the anterior zone of a slug lifts off the agar, some anterior-

like cells on the ventral side of the slug form a stationary pile at the base of lifted anterior zone (so these cells move relative to the posterior of the slug as the slug continuously migrates forward). Furthermore, when the tip lands on the agar, ventral anterior-like cells suddenly start to migrate chemotactically toward the landing point to form a new pile at the base of lifting anterior zone (Dormann et al., 1996). In this case, the landing point appears to become a secondary cAMP source that does not interfere with the preexisting tip. This secondary cAMP source was also indirectly detected at the same position within the slug as the source of Ca^{++} waves (Cubitt et al., 1995).

How such secondary organizing centers come into being and how they are managed is currently unknown, but they might be controlled by cell-type specific differences in cAMP receptors (Dormann et al., 1996). In the case of light irradiated flattened slugs, the initial light response occurred only in the active cells on the periphery (**Fig. 30, 31**). Accordingly, light-induced cAMP emission may affect only a subpopulation of cells within the slug anterior zone. Such a light stimulus does not completely disrupt the slug morphology but causes the bending of anterior zone.

5.2.3 A possible mechanism of light induced cAMP release

Although there is no experimental clue as to the mechanism of light induced cAMP emission at present, a possible mechanism can be proposed based on known facts. Three different types of adenylyl cyclases are active during the multicellular stages of *D. discoideum* ; ACA, ACG and ACB (ACR). ACA is an aggregation phase cyclase and its activity decreases after aggregation. ACG activity is present only during culmination phase (Pitt et al., 1992). ACB is a recently discovered adenylyl cyclase that peaks in activity during the slug stage (Kim et al., 1999; Meima and Schaap, 1999b; Soderbom et al., 1999) and could be responsible for cAMP formation during phototaxis. ACB

is a part of two component signal transduction system, which uses a histidine kinase to transfer phosphate from a phosphoprotein intermediate to target proteins. Such systems have been conserved from bacteria to vertebrates and are often used to regulate responses to environmental stimuli (Loomis et al., 1998; Meima and Schaap, 1999a). In the absence of environmental stimuli most of the cAMP produced by ACB seems to be hydrolyzed, probably because ACB colocalizes with a cAMP-specific phosphodiesterase (REGA; Meima and Schaap, 1999a). Genetic evidence indicates that REGA activity is controlled by an upstream regulator histidine kinase DHAC via a phosphotransferase RDAE. Interestingly, DHAC was discovered as the regulator that links environmental triggers (overhead light, depletion of surrounding ammonia and humidity) to the onset of culmination (Singleton et al., 1998). Suppression of DHAC activity leads to an increase in intracellular cAMP via suppression of the phosphodiesterase activity of REGA; increased levels of cAMP then activate cAMP dependent kinase (PKA) and the expression of genes required for the culmination (Singleton et al., 1998).

During slug phototaxis, the emission of cAMP following light irradiation could be mediated by suppression of REGA cAMP-phosphodiesterase activity. The resulting increase in intracellular cAMP could be secreted to the extracellular space (Dinauer et al., 1980a; Schaap et al., 1995). The catalytic domain of ACB is similar to the catalytic domain of adenylyl cyclase of cyanobacteria *Spirulina platensis* and *Anabaena spirulensis* (Soderbom et al., 1999) and the activity of these bacterial adenylyl cyclases is known to be under the control of photoreceptors (Kasahara and Ohmori, 1999; Katayama and Ohmori, 1997). Further investigation of the effects of light on this phosphorelay signal transduction pathway in *Dictyostelium*, including the activity of ACB, is thus of particular interest.

In many organisms such as *E. coli*, cyanobacteria, *Arabidopsis* and vertebrates, the phototransduction pathway involves the activation and the

inactivaton of phosphodiesterase activity by binding of cGMP to phosphodiesterase (Aravind and Ponting, 1997). Whether light induced cAMP secretion in *Dictyostelium* is subject to such regulation is still an open question. However, a light-induced cGMP transient has been detected by Darcy *et al.* (1994) and could be upstream of the light induced cAMP release pathway. Light-induced cGMP transients peak at 1 min after light stimulation and return to basal levels after 5 min. Present results suggest that the light induced cAMP secretion occurred after the light-stimulated intracellular cGMP transient because release of cAMP to the extracellular space was first detectable ca. 10 min after light irradiation (**Fig. 35**).

5.2.4 Decreased up-down motion is probably due to light induced cAMP secretion within the slug tip

The analysis of phototactic behavior showed that slugs increased speed and length during phototaxis, both of which are likely a result of a decreased frequency of up-down motion. Although the analysis of slug behavior did not directly indicate how a slug decreases its frequency of up-down motion, a possible mechanism could be that light induced cAMP secretion interferes with the up-down motion of the slug tip. Mathematical modeling of slug migration based on cAMP signaling and cell movement resulted in a slug migration pattern which included a periodic up-down motion of the slug tip (Bretschneider et al., 1995; Bretschneider et al., 1999). The simulations showed that the up-down motion is a consequence of the asymmetry of the rotation speed caused by cells following a scroll-shaped cAMP wave. For example, faster rotation in the ventral side caused the slug tip to go down to the agar surface. In this context, the observed up-down motion could be regarded as the vertical mode of anterior-tip bending due to the asymmetry of cell movement in the dorso-ventral axis. If asymmetric cAMP secretion and asymmetric accumulation of cells is induced by light, this may decrease the up-down motion.

Secretion of cAMP following light irradiation may also affect the slime sheath of the slug. The slime sheath appears to play a role in slug phototaxis since mutants with defects in sheath synthesis, *ecmA*-, exhibit alterations in morphology and impaired phototaxis (Wilkins and Williams, 1995). Mutants lacking *ecmA* form normal slugs, but the slime sheath is mechanically labile and mutant slugs show delayed compaction of the tail (Morrison et al., 1994). Expression of *ecmA* is confined to the slug tip (Jermyn et al., 1989) and has been shown to be stimulated by light irradiation (Jermyn and Williams, 1991) and also by cAMP (Berks and Kay, 1990). If light increases cAMP secretion and subsequently enhances ecmA synthesis, the sheath could be "stiffened" and mechanically support a prolonged lift of the tip during phototaxis (**Fig. 12, 13**).

5.2.5 An increase of speed in the posterior zone is due to thinning of the slug diameter

An increase in slug speed ultimately requires an increase in the speed of all cells in the posterior zone. This could be induced either by a change in cAMP signaling dynamics or by changing the diameter of the slug. A change in chemotactic signaling is unlikely since the speed of cAMP wave propagation is not consistent with the delay of one hour in speed change at the posterior end (Breen and Williams, 1994; Durston et al., 1979)[4]. On the other hand a light induced decrease in slug diameter could explain such a delay. Light irradiation initially leads to a thinning of the anterior zone and finally to a decreased diameter of the whole slug (**Fig. 15, 19**). The decrease in the diameter would lead to an increase in the flow of cells and hence an increase in the speed. The slug shown in **Fig. 12** had an average speed of

[4] Durston & Vork (1979) estimated that waves of movement propagate along the slug axis at a rate of 2.9 mm/hr. Breen & Williams (1994) reported 4.3 mm/hr for the propagation speed of waves within the slug.

1.30 ±0.33 mm/hr and an average length of 1.30 ±0.06 mm in the dark. The posterior end first increased in speed 60 minutes after the tip, indicating that the cells at the posterior end increased speed when they reached the position where the diameter of the tip decreased. Since the slime sheath is stationary relative to the substrate, posterior cells move through sheath which was laid down by anterior cells. If light irradiation decreased sheath diameter in the anterior, then the posterior cells would increase in speed as they migrate into the narrowed sheath. For posterior cells in the slug in **Fig. 12** this occurred after 60 min. Light stimulation of ecmA synthesis may further increase the mechanical rigidity of the slime sheath and support the decreased sheath diameter.

5.3 New model of phototaxis

Based on the results presented here, a new model of slug phototaxis can be proposed. When a slug is irradiated by unilateral light, a lens effect increases light intensity on the distal side and induces local cAMP release (**Fig. 41a**). Then a population of cells, which are competent to sense this cAMP signal, move chemotactically toward the source of cAMP emission. As a result, cells asymmetrically accumulate on the side distal to the light source thus bending the slug tip toward the light source (**Fig.41b**). Because asymmetric illumination induces cell accumulation on the distal side, the slug tip repetitively corrects its orientation by serpentine motion during phototaxis. The light-induced secondary cAMP source also affects overall slug behavior (**Fig. 41c**). It attenuates the tangential rotation of the prestalk cells and hence decreases the up-down motion of the tip. Decreased frequency of the up-down motion allows a prolonged elongation of the anterior zone. As a result, the tip proceeds at a faster speed while the acceleration at the tail is delayed. Accordingly, the overall slug length increases and slug diameter decreases. Thinning of the slug increases the flow rate of cells in the slug posterior zone and finally accelerates the tail

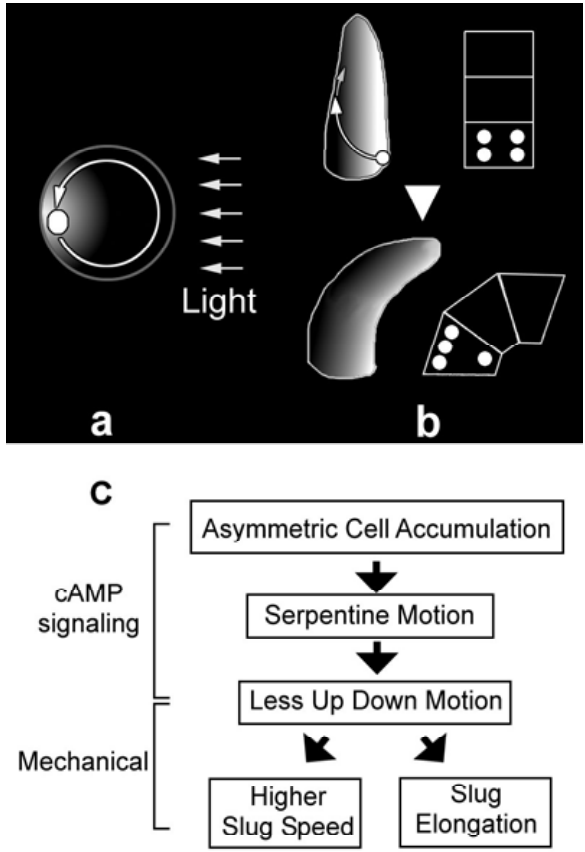

Figure 41. A New Model of Phototaxis.

(a) Cross section of slug anterior zone. Upon light irradiation, cells release cAMP on the side distal to the light source, where a lens effect focuses the light. (b) Asymmetric cell accumulation. Wire frame diagram on the right side schematically shows the effect of asymmetric cell accumulation on the shape of slug anterior zone. (c) Effect of light irradiation on slug behavior and morphology. Serpentine motion and less up down motion of the tip are both the consequences of asymmetric cell accumulation i.e. cAMP signaling. Higher speed of slug movement and elongation of slug are both the mechanical consequence of less up down motion of the tip.

speed. Since this new model depends largely on cell-cell signaling, it becomes evident why phototaxis is pronounced in the multicellular stage but not in single cells.

5.4 Ammonia and slug speed

Ammonia has been proposed as a factor that is involved in slug turning during phototaxis (Bonner, 1994). According to this view, ammonia is emitted on the side away from light source and increases cell speed there, resulting in turning and an increase in slug speed (Bonner et al., 1988). In contrast to Bonner's results, the results in this thesis showed a steady decrease of slug speed with increasing partial pressure of ammonia (**Fig. 23**; Bonner et al., 1988). Since slug movement is organized by cAMP signaling, this result is consistent with previous observations that ammonia attenuates cAMP signaling (Siegert and Weijer, 1989; van Haastert, 1985; Williams et al., 1984). Thus, it seems unlikely that emission of ammonia is responsible for the increase in slug speed observed in the phototaxis experiments[5] (**Fig. 12, 13**).

[5] It should be noted that the half-inhibition of slug speed occurred by ca. 0.0025 mmHg ammonia (= 1.6×10^{-7} M; **Fig. 23**); on the other hand, considerably lower ammonia concentration, as low as a gradient of 4×10^{-14} mol / mm, induces the turning (Kosugi and Inouye, 1989). The role of ammonia in the phototactic turning should be left as a further question.

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not of what the network describes.

L. Wittgenstein,
Tractatus Logico-Philosophicus

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7 Abbreviations

cAMP	cyclic adenosine 3’5’-monophosphate
cGMP	cyclic guanosine 3’5’-monophosphate
STF	slug turning factor
ACA	adenylyl cyclase A
ACB	adenylyl cyclase B
ACG	adenylyl cyclase G
$[Ca^{++}]_i$	cytosolic free calcium concentration
2D	two dimension
3D	three dimension
PKA	cAMP-dependent kinase
Rm	regulatory subunit of cAMP-dependent kinase
GTP	guanosine triphosphate
GSK3	glycogen synthesis kinase 3
RNA	ribonucleic acid
EGTA	ethylene glycol bis(beta-aminoethyl ether)-N,N,N’,N’-tetraacetate
KF	potassium fluoride
SB	Sussman broth
CCD	charge coupled device
EP	efficiency of phototaxis
DiR	1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (= $DiIC_{18}(7)$)

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