Investigating the Transition to Multicellular Life on Small Scales by Physically Modifying a Chemical Signaling Channel

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The study of the starvation response of the social amoeba system *Dictyostelium discoideum* has long been regarded as an important opportunity to learn about the transition to multicellular life for eukaryotic organisms. Here we focus on two critical developmental steps: the first indication that a communications network between formerly independent cells has been established as evidenced by synchronized periodic shape changes and the first stages of aggregation as indicated by the appearance of streams of migrating cells. In contrast to earlier work which has stressed large scale structures, to be interpreted with continuum theories, we focus our attention on the smallest scales of interest for multicellular effects. In order to better understand the means by which these developmental steps come about we study their timing from the start of starvation as a function of a hitherto underappreciated experimental variable: the thickness of the aqueous layer through which the chemical messages diffuse between the cells while the cells themselves crawl about on a substrate. We also explore the importance of sparseness in such microbial life by examining these transitions as a function of the substrate's coverage by cells, again with particular attention to the smallest scale multicellular features. In contrast to earlier theoretical approaches that rely on chemical signaling in a two dimensions, we compare these measurements with a

theory that explicitly takes into account the discreteness of life and the transport of chemical signals through a three dimensional layer of finite thickness. Despite the large fluctuations that are expected for small scale observations of these heterogeneous systems, we observe a significant speedup to synchronized pulsation when the signaling layer thickness is less than a micron when compared to thicker layers. Our rough theory which focuses on the time that it takes a collection of oscillators to synchronize gives qualitative insight into this result. Turning to the time required for stream formation: we see an unexpected reduction in the time to pass to this stage of multicellular development in bulk vs. thin film systems. Finally, we report how small collections of cells (``squads'') universally form for all densities we examined through contact accretion but do not manage, as one might expect at low surface densities, to outrun long distance chemical signaling as a means of building streams.

I. Introduction

Over several exciting hours, starving colonies of the eukaryote *Dictyostelium discoideum* (henceforth referred to here as Dicty) form themselves into a genomic lifeboat containing the nuclear material of up to 10^5 cells[1]. The very first indication that these colonies have made the jump to multicellular life is the readily recognizable appearance of synchronized periodic shape changes over patches of adjacent cells, typically involving as few as tens of cells. The question of how quickly Dicty colonies

are able to bring about this first collective behavior on the smallest length scales will be the focus of this work. These changes in cell morphology are responsible for the vivid large scale patterns seen in dark field microscopy of colonies on substrates[2] and in the periodic light scattering from suspensions.[3] It has long been understood that such shape changes in the form of ``cringes''[4] indicate the reception of external chemical signals, in the form of cyclic adenosine monophosphate (cAMP) molecules. The subsequent emission of the same signals is understood to be the means by which a signaling network is established in collections of cells.[5] In order to better understand the establishment of such multicellular life, we sought to perturb the signaling channel itself by restricting its vertical height (h).[6] In the following section we will describe the various preparations we used to accomplish this. We will also describe the starvation protocols that we followed and the manner in which the system was observed, always with the emphasis on small scale behavior. In choosing between the many possible visual observables, we focused on two landmark developmental events: the earliest time (T_P) (from the beginning of starvation) we noticed synchronized cell shape pulsing and the earliest time (T_S) we noticed the coalescence of migrating cells into streams. Steams need not be continuous. In fact we frequently observed streams composed of only a few cells. As has been recently noted,[7] these small scale aggregates readily move as a unit. In fact at first glance they appear to be single cells. We christen these broken streams with the name ``squads.'' We noted whether squads were present in the various preparations used. Besides the thickness of the aqueous layer, we also measured a surface density variable, f, the fraction of the substrate that was covered by cells. This is of particular interest as we focus our attention on small scale features in contrast to the large scale averaging of

cellular life in earlier work that stressed wave signaling. Hence we are especially interested in the sparse regime, i.e. low f. After presenting the observations, we will provide a rough theory for T_P for the establishment of a system of synchronized oscillators from out of a collection of cells that spontaneously emit randomly-timed bursts of signal molecules. We will then compare this theory and experiment for the time to synchronized pulsing, Next, the observation of the early stages of streaming (at the time given by T_S) are given and comparisons are made with a rough theory. We then give a discussion of the role that squads play in the formation of large scale aggregates.

II. Experimental Methods

1. Specimen Preparation

In order to study the consequences of vertical confinement of the chemical signaling channel for colonies of starved Dicty cells on a horizontal substrate we prepared signaling layers in the different ways illustrated in Figure 1: as aqueous wetting layers of different thicknesses and as soaked hydrophilic highly porous membranes.

A. Wetting Layer Preparation (Figures 1a and 1b)

In our first approach we use aqueous wetting layers on glass substrates as signaling layers. These layers were of three types: ultrathin (less than 1 micron thick), thin (ranging from 16-42 microns but mostly in the 20 to 30 microns thick range) and bulk (approximately 2000 microns thick). The procedures which we followed for cell culture and starvation (including the composition of the aqueous media used) based on standard Dicty protocols[8] were as follows: we grew our cell lines at 19-21 deg C using 25 ml rotary shaken suspension culture of the axenic cell line AX3 (the strain used was

Wf38 in AX3[9]) with HL5 culture media. Cells were harvested from the exponential growth phase and concentrated through three rounds of centrifugation and exchange of media with development buffer (DB) prepared according to Ref. 10. The essential property of DB is that while it provides appropriate salt concentrations for life, it is missing nutrients, thereby starving the system and inducing development. Our concentration procedure produced cell densities up to the 10^7 cell/ml range, as determined by dilution and subsequent slide based cell counting. For substrates for starvation we used plasma-cleaned[11] glass coverslips commercially glued over holes in plastic Petri dishes.[12] The concentrated suspensions were diluted in order to achieve the desired cell density. In order to produce thin film specimens, 1 ul of the starved cell suspension was applied to the centers of the dishes. Immediately a 1.0 ml volume of DB-saturated[13] light mineral oil[14] was then applied to prevent the preparation from drying out. This preparation resulted in large area (typically 33 mm^2) roughly circular pancake-shaped droplets. The thicknesses of these structures were gauged by measuring their area by matching them with a lineup of digitally drawn circular and elliptical shapes of various areas and ellipticities. From the volume used, we compute the layer heights. These ranged from 14 to 42 microns,[15] with most specimens in the range of 20 to 30 microns.

In order to prepare ultrathin films, we added one main additional step: a cotton thread with a DB-saturated tip was inserted through the mineral oil overlayer in order to make contact with the droplet which was typically 5 ul in volume.[16] We noticed that the droplet would quickly lose visual contrast, indicating that DB was being wicked away. In some cases, the wick was eventually removed. In others, it remained. We argue based on experience with binary liquid mixtures near hydrophilic substrates that what remained on

the glass was an exceedingly thin film of the aqueous phase of less than a micron in thickness.[17] It might seem surprising that Dicty could function in such an environment where except for the substrate, its surroundings are almost entirely nonaqueous. But our preparation recalls the work of Bonner[18] when he produced ``two-dimensional slugs'' that adhered to a glass substrate by inserting developed aggregates of starved Dicty into a bulk phase of mineral oil.

For our bulk specimens, in place of the droplet of cell suspension we used a 200 ul volume of the starved cell suspension before applying the mineral oil rich overlayer. We typically prepared several thin layer specimens in a batch along with a bulk specimen. In a typical run, we selected one thin or ultrathin sample for up to more than 24 hours of observation. Typically we selected for the greatest uniformity in spatial distribution of cells. To summarize, we produced reduced-thickness signaling layers for starved Dicty cells on glass substrates by forming wetting layers. We also used bulk phase specimens. We observed preaggregation behavior as a function of cell surface density. On occasions where aggregation occurred, we observed the expected[19] formation of mounds and ultimately stalks and fruiting bodies, which grew up readily through the mineral oil rich overlayer.

B. Membrane Preparation (Figure 1c)

As a second means of constructing a confined signaling channel, we prepared as an alternative to the glass substrate, beds of thin DB-soaked hydrophilic porous membranes,[20] thin layer analogs to the familiar thick filter paper substrates used in Dicty starvation development studies.[21] Our membranes had a pore size of 0.1 microns, which is too small for the cells (which are typically 10 microns across) to penetrate. The idea

was that cells would signal by diffusing signal molecules through the membranes just as they would communicate through the wetting layers in the other preparations. We achieved this configuration by placing the cell specimens on top of one or two membranes that were injected with DB. The membranes were 30 microns thick. In this manner, we provided signaling channels with a single membrane of 30 microns thickness and with a pair of membranes on top of each other we had vertical confinement for 60 microns of height. The details for our thin membrane preparations were as follows: the same type of glass-bottomed Petri dishes used in wetting layer preparations were plasma cleaned as in the wetting layer preparations. For a single layer membrane preparation we proceeded as follows: membranes which had been cut into typically 8 by 7 mm rectangles were placed on the glass substrate. We used a limited amount (0.5 ul) of cell suspension in DB so as not to produce an uncontrolled excess, which would ruin our establishment of a controlled thickness signaling layer. We verified this condition by noting that the wetted area (typically 6 mm across) of the membrane did not cover it entirely. As before a mineral oil rich overlayer of 200 ul volume was gently applied on top of all this. For two layer membrane preparations, the protocol was the same, except that a single membrane was initially placed on the substrate and a volume of 0.5 ul of DB was used to wet it before the above procedure was repeated for the second membrane. We found that fruiting bodies atop stalks could be observed to form with these membrane preparations.

2. Observation Methods

   Time lapse (10 seconds delay between frames for thin and ultrathin films, 20 sec for membrane preparations, and 10 sec to 1 minute for bulk systems) recordings were

made of the thin, ultrathin and membrane preparations using inverted microscopes,[22] over a viewing area of 870 by 660 microns. This small area (about 0.6 mm^2) being a factor of 1500 smaller than in typical wave signaling studies,[23] allowed us to sample the behavior of the system on the scale of individual cells. A sample frame is shown in Figure 2. For the bulk specimens, observations were made using a noninverted microscope[24] at the same time as the recordings of a confined layer preparation. For all preparations, early frames were printed out, highlighted for cells manually and then processed with the ImageJ[25] program in order to ascertain the fraction of the substrate that was covered by cells, f. From repeated measurements of the same image, we estimate that our relative uncertainty in f is about 6%. The videos were viewed at factor of 150 speedup or faster (checked as needed by different observers) to note synchronized shape changes (T_P) of collections of cells. We also noted the earliest time for streaming (T_S) and whether small adherent groups of cells (squads) were present. From repeated observations of a typical video, the uncertainty in establishing T_P is 0.2 hour and for T_S is 0.1 hour. Individual cells merged tightly to form squads such that cell boundaries were not discernable. However, we could still differentiate large single cells from squads by witnessing their formation, as seen in Figure 3 where a cell is joining a squad; or dissociation, where cells are seen shedding from a squad. The process of formation and destruction of squads were observed to be continuously ongoing.

III. Observations of Earliest Signs of Synchronized Pulsing

Figure 4 below shows our observations of the first signs of collective pulsing as a function of signaling layer thickness and fraction of the substrate covered by cells for bulk, thin and ultrathin systems. We found that for the shaded ultrathin points there was some deviation of interest from the others: either the thread was left in the contact with the film, or the starved preparation was left in the bulk phase for an extended period of time. Figure 5 gives this same information for the membrane preparations. We note that within the uncertainty in T_P, the results for thin films and bulk preparations are indistinguishable. The scatter in results is considerable and much greater than our uncertainty in establishing the onset of synchronized pulsing in a single run (0.2 hour). The relative uncertainty in f, 0.06, is insignificant in this and all our other measurements. Despite the scatter in the ultrathin film results, we see a good indication of a reduction in T_P around 25% coverage in the ultrathin film results compared to the bulk and thin film observations. We also note that there is a threshold for collective behavior apparent in the bulk data as follows. After about 17 hours of observation, no synchronized pulsing was in evidence for f at 2% or lower. We compare this to the minimum surface density for aggregation of 10^5 cells/cm^2 reported for the AX2 strain.[26] Assuming that Dicty cells settle into random positions on the substrate and have an approximately circular footprint with a 5 micron radius gives this as a fractional areal coverage of 7.5%. We speculate that the deviation from our results might be attributed to the different strains being compared here or uncertainty in the cell size.

The membrane measurements shown in Figure 5 tell a very different story: in contrast to the agreement of thin film and bulk data in Figure 4 the one and two layer

membrane systems show a surprising trend toward shorter time to synchronization for larger signaling channel thickness. We speculate that this might be because the transport of the signal molecules through the membrane is not by bulk diffusion alone, but involves surface diffusion on the porous membrane material in a manner that drastically differs from the simple diffusive transport through the open signaling layer liquid phase of the wetting layer systems.

IV. Theory for Earliest Signs of Synchronized Pulsing and Comparison with Experiment

We now develop a rough prediction for the time for synchronized pulsing T_P given that starvation development has proceeded far enough to produce a collection cells which are of spontaneously emitting bursts of signal molecules and can be triggered into oscillation by the combined output signals of their neighbors. In this manner, we emphasize the discreteness of cellular life on small scales, in contrast to the continuum theories that are restricted to considering large scale signaling wave patterns in sufficiently dense systems.[27] We expect that the cells are employing short pulse signaling since it has been observed[28] in suspensions of synchronized cells that the duration of extracellular cAMP emission is short compared to the oscillation period.[29] Recognizing that entrainment does occur, we focus attention on the additional time required to bring it about, once the required biochemical signaling apparatus is in place. For the sake of simplicity, we assert that an individual cell's development time to spontaneous signal emission and signal reception, T_{DEV}, is fixed for all conditions we explored (i.e.

aqueous layer thickness and the substrate surface coverage). Our aim therefore in this section is to predict the time to establish entrained oscillations in a collection of cells T_{SYNC} in order that we can find the observed time to synchronized shape changes, T_P through

$$T_P = T_{SYNC} + T_{DEV}.$$ **(1)**

We will treat the problem self-consistently as follows: imagining the situation from the view of a single potential oscillator in the collection, one must synchronize[30] to the combined signals of the other signal sources in the collection, which we will regard as a master signal. We argue that in this pre-entrainment period, there is no need to invoke the notion of a pacemaker cell[31] to break the phase symmetry of the combined signal of the collection of neighboring cells, but rather a transient signal with sufficient strength at the required frequency emerges from the randomly phased combination of burst signals from neighboring cells. Just as one expects a randomly phased collection of steady oscillators to yield a combined signal with amplitude which grows as $N^{1/2}$ where N is the number of individual oscillators,[32] we expect that the N cells in the collection that will eventually synchronize will produce a fluctuating chemical concentration signal with a component at the frequency of interest whose strength will scale as $N^{1/2}$ initially.[33] Following Strogatz's[34] discussion of the entrainment of a slave oscillator to an imposed master we model this lock-in phenomenon as a correction of the master-slave phase error, ϕ as follows:

$$\dot{\phi} = -\gamma \sin(\phi) . \qquad \text{(2)}$$

Where γ represents the strength of the master signal impressed on the slave.

Simplifying, we consider only the characteristic exponential decay limit when the slave

cell is close to synchronization. We therefore expect that the desired time to

synchronization is given by T_{SYNC} through the relation

$$T_{SYNC} = \gamma^{-1} . \qquad \text{(3)}$$

We can now express N as a function of the characteristic spatial extent of the collection

on the substrate, L, the fractional substrate coverage, f, and the radius for each Dicty

cell on the substrate, a as follows:

$$N = f (L^2 /(\pi a^2)). \qquad \text{(4)}$$

In our pursuit of the earliest signs of synchronized pulsing, we need to be aware that L

does not represent the size of the collection of cells that will ultimately be swept up into

the final aggregate. This ultimate size can be drastically adjusted by shedding groups of

cells well after the initial aggregate is formed by large scale streaming that follows the

initial period of synchronized pulsing that we are studying. Such later stage size

regulation has been extensively explored.[35] Bearing this distinction between the

collection of cells that initially signal each other and those that ultimately form an

aggregate in mind, we make the simplest possible assumption here and regard the

collection size L for initial synchronized pulsing as being independent of preparation,

i.e. independent of both initial fractional surface area coverage (f) and signaling layer

thickness (h). We now have a simple way to scale up (through our $N^{1/2}$ law) the signal strength due to a single cell. We need $A_0(\omega)$, the spectral component of the chemical emission strength from each individual cell at the frequency of the Dicty shape oscillations (ω). We therefore assert that in Equation (2) we have the following expression for the phase correction strength

$$\gamma = 1/T_{SYNC} \propto N^{1/2} A_0(\omega). \quad \text{(5)}$$

We now need to find the time course of the signal produced by a single cell. For this we consider the concentration field that results from a point source releasing a unit dose of signaling molecules at an instant of time. Our signaling media are slab shaped aqueous phases with thickness h with no flux at both the bottom and top surfaces. We therefore turn to the concentration field due to an appropriate string of point image sources36 spaced at intervals of $2h$ above and below the actual source along a line (the z axis) which is perpendicular to the substrate. Placing the source at the origin on the substrate (x = y = z = 0) we have the following expression for the concentration field for a point release of unit concentration at time t = 0: (In the following expression l ranges over all integers).

$$c(t, x, y, z, h) = 2 \sum_{l=-\infty}^{+\infty} \frac{\exp[-(1/4)\frac{((z+2hl)^2 + x^2 + y^2)}{(Dt)}]}{(4\pi Dt)^{3/2}} \quad . \quad \text{(6)}$$

In this expression D is the diffusivity of the signal molecule. Since we are only interested in the signaling to other cells on the substrate, we can take z=0 in the above expression and for convenience, define $r^2 \equiv x^2 + y^2$.

We therefore have

$$c(t,r,h) = 2 \sum_{l=-\infty}^{+\infty} \frac{\exp[-(1/4)\frac{((2hl)^2 + r^2)}{(Dt)}]}{(4\pi D t)^{3/2}} . \qquad (7)$$

For the purposes of synchronization we must consider the received signal strength at the oscillatory frequency. This is provided by considering the Fourier transform of $c(t,r,h)$ at frequency ω. We therefore have:

$$A_0(\omega) \propto \int_0^\infty \sin(\omega t) \, c(t,r,h) \, dt . \qquad (8)$$

Since we will ultimately compare the signal strength for different signaling channel thicknesses with that in the bulk we need not be concerned with knowing either the amplitude of the transmitted signal at the source or the receiver sensitivity since these quantities will not appear in the desired ratio. Combining Eqns. 7 and 8 we have

$$A_0(\omega) \propto \sum_{l=-\infty}^{+\infty} \int_0^\infty \sin(\omega t) \, \exp(-\gamma_l / t) t^{-3/2} dt . \qquad (9)$$

Where

$$\gamma_l \equiv (1/4)[(2hl)^2 + r^2]/D. \qquad (10)$$

From Ref. (37) we have for the integrals in Eqn. 9:

$$A_0(\omega) \propto \sum_{l=-\infty}^{+\infty} \gamma_l^{-1/2} \exp[-(2\gamma_l \omega)^{1/2}] \, \sin[(2\gamma_l \omega)^{1/2}] \qquad (11)$$

In order to further simplify matters, we assign r to be the typical signaling range given by the average spacing between cells on the substrate: therefore, in place of r we will use

$$\langle r \rangle = (f/(\pi a^2))^{-1/2} \qquad (12)$$

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We see that it is natural to introduce

$$\theta_l \equiv (2\gamma_l\omega)^{1/2} \qquad \textbf{(13)}$$

We now consider the ratio of characteristic synchronization time T_{SYNC} for a signaling layer of height h normalized to that of an infinitely thick layer ($h = \infty$) (i.e. to represent signaling in the bulk) with the substrate completely covered with cells ($f = 1$). That is to say, we wish to know the ratio:

$$T_{SYNC}(h, f) / T_{SYNC}(h = \infty, f = 1).$$

Now, since $\theta_{l \neq 0}(h = \infty) = \infty \qquad \textbf{(14)}$

we see that for $h = \infty$, the $l \neq 0$ terms in Eqn. (11) vanish.

Combining Eqns. (4, 5, and 11) and noting the symmetry $\theta_l = \theta_{-l}$ we find:

$$T_{SYNC}(h, f) / T_{SYNC}(h = \infty, f = 1) =$$

$$\frac{f^{-1/2}[Z_0(f = 1)]}{[Z_0(f) + 2\sum_{l=1,2,3,...} Z_l(f, h)]} \qquad \textbf{(15)}$$

Where, using Eqns. (10, 11, 12 and 13) we have employed the functions:

$$Z_l \equiv (1/\theta_l)exp(-\theta_l)sin(\theta_l) \qquad \textbf{(16)}$$

Which can simplify as follows:

$$\theta_l = \mu_0 f^{-1/2} [\frac{4h^2l^2f}{\pi a^2} + 1]^{1/2} \qquad \textbf{(17)}$$

by introducing

$$\mu_0 \equiv (\frac{\omega a^2 \pi}{2D})^{1/2}. \qquad \textbf{(18)}$$

In order to evaluate these expressions we used the following values: for D,

444 micron^2 per second,[38] for a, 5 microns[39], and to find the angular frequency of

oscillation ω, we used a period of 7 minutes.[40]

In order to produce the predictions[41] drawn as lines in Figure 4, we treated

T_{DEV} and $T_{SYNC}(h = \infty, f = 1)$ as adjustable parameters, finding that the bulk data

of Figure 4 constrains these quantities to be about 1.5 and 1.0 hours respectively. Note

that this value for T_{DEV} is somewhat short compared to the quoted value of 5 hours for

the development of the essential cAMP receptor car1.[42]

Considering the theory in Figure 4, we see that a focusing effect is predicted due

to confinement: as the channel thickness decreases, there is a quickening of the

synchronization process. We also see hastening expected as the surface coverage

increases. The theory predicts that bulk behavior is attained by any layer thickness greater

than 500 microns (the results for 500 and 1000 microns are indistinguishable). Thus we

were justified in fitting our bulk data (where we have a value of h of about 3000

microns) to give $T_{SYNC}(h = \infty, f = 1)$. The theory predicts that on the scale shown

the ultrathin film synchronization should be immediate and therefore independent of

surface areal coverage, in agreement with the experimental results in Figure 4. The theory

does however miss an essential feature of the observations. While the theory predicts that

the thin film (generally 20-30 microns thick) and bulk results should be distinguishable

even allowing for our experimental scatter, they are observed to be the same. Considering the threshold we detected for aggregation at 2% surface areal coverage, we see from our plots that this might be indicative of the predicted failure to synchronize in the time over which the observations were made.

Turning to our membrane results shown in Figure 5, we see that the striking feature that we noted earlier, namely the hastening of the time to synchronization with increased signaling layer thickness is at odds with our theory. Again, we speculate that this unexpected result might be due to the special possibilities for surface transport afforded by this porous medium. To summarize, our rough theory provides semi-quantitative agreement with the measured time to collective pulsing with our wetting layer experiments, and reinforces our surprise with the membrane results.

V. Observations and Theory of the Time Interval between the Onset of Synchronized Pulsing and Streaming

In Figure 6 we show the observed time interval between streaming and collective pulsing in bulk, thin and ultrathin films. There is considerable scatter (much greater than the uncertainty we noted in establishing the streaming and collective pulsing times in a sample run) in the thin and ultrathin film results, which are thereby indistinguishable. We see that the bulk values by contrast are considerably smaller, especially at the lowest fractional areal coverage. In our simplest conception of the problem, we imagine that the process by which cells coalesce into streams would be by direct contact and hence make no further use of the long range signaling system. This is apparently not the case since the layer thickness matters. Turning to our membrane preparations, by contrast Figure 7

shows the expected thickness independence when we compare results for different

membrane sizes. For a given surface areal coverage, the coalescence into streams occurs

as quickly on membranes as we observe it to do on glass under bulk aqueous phases.

We can give a rough prediction of the dependence on substrate coverage of the

time delay between collective pulsing and coalescence into streams as follows:

presuming the cells move at a fixed speed (s) and that they have swept together by

accretion a critical line density of cells to form the first branch of a stream, then we can

ask over what time we might we expect this to achieved, given a surface density of cell

set by the fractional areal coverage. If we let l represent the average spacing of cells, then

we expect that:

$$T_S - T_P = l / s \qquad \textbf{(19)}$$

And since we expect

$$l \propto f^{-1/2}. \qquad \textbf{(20)}$$

We predict

$$T_S - T_P \propto f^{-1/2}. \qquad \textbf{(21)}$$

We see from Figure 6 that while the sparseness and scatter in the thin and ultrathin

observations do not allow for a test of this ``sweep'' theory prediction, the bulk results

appear to rule it out (from our repeated measurements of T_P and T_S on the same video

our uncertainty in $T_S - T_P$, 0.2 hr., is much smaller than the scatter we see in Figure 6).

Turning to our membrane observations, Figure 7, again with limited data, shows

the agreement we had expected between one and two membrane results and this rough

theory.

VI. Observations of Few Cell Aggregates (``Squads'')

Our observations of squads (see Figure 3) are simply summarized. We found them to be present in all cases observed. We had expected that they would serve as an alternative means of aggregation when densities were too low to enable effective long range chemical signaling. Had this been the case, we would have seen streaming without synchronized pulsing preceding it. This was not the case in our observations. Upon reflection, we can ask at what fractional areal coverage the mutant strains of Wang and Kupsa[43] were able to achieve aggregation with the cAMP signaling apparatus disabled (specifically the apparatus for external cAMP secretion (the ACA transmembrane protein) is defective) we estimated that this was essentially 100%, a higher density than we explored.

VII. Summary and Outlook

We have presented here a first look, both experimentally and theoretically, into the effect of physically modifying through height control the chemical signaling channel seen as essential to bringing about aggregation as a consequence of starvation in the social amoeba system *Dictyostelium discoideum*. We have observed by noting the first indications of synchronized oscillations in cell shape the establishment of a communications network through chemical signaling. We have noted an apparent speedup when one passed to the thinnest signaling channels we have prepared, in qualitative agreement with a theory we have developed which treats the system in a manner that is able to address the sparse coverage of cellular life in the situations that are

of the greatest biological challenge to this system. We were surprised by the qualitatively

unexpected results we encountered in preparations which altered the thickness of the

signaling channel through the use of porous membranes. This is particularly significant

since starvation experiments are often performed on porous substrates. This suggests that

we need to better understand the passage of chemical signals in such systems. Our

observations invite exploration with larger fields of view in order to go beyond the first

signs of collective life that we have focused on here. This will permit one to pass from

these small scale collective features to what will ultimately be the catch basins for all the

cells that will form a single aggregate. In this manner one could expect to relate these

small scale structures to large scale observations.[44] We have argued that by stressing

small scale features, we may have detected significant intrinsic fluctuations. Therefore a

more extensive sampling than we have completed would provide vital information for

such spatial noise effects. It would also be important to independently establish the time

that is required to initiate cell signaling. In order to achieve this, one can look forward to

the use of fluorescence reporter experiments that indicate the biochemical state of the

signaling apparatus. We also look forward to digitally automating the process by which

video is examined for dynamic features in order to identify aspects of collective order.

On the theoretical front, our rough theory for the time to the first signs of multicellular

life based on the time to the formation of synchronized cell pulsations could well be a

proper first step in the spirit of dimensional analysis toward a detailed explanation. We

expect that the results we have predicted would be valid even if the means by which

collective oscillations began were very different. For example an alternative route could

be through the synchronization of randomly phased cells which are already experiencing

self-oscillation as opposed to the mechanism of a collection of cells randomly releasing shorts bursts of signal molecules that we have presented here. We feel that the exploration of surface modes of transport in the porous membrane systems might lead to an understanding of the surprising behavior we have encountered with membrane systems when we consider the time to collective pulsing. We also welcome more refined stochastic approaches that feature the effects of the discreteness of cellular life.

Turning to the coalescence into streams: the importance of the thickness of the chemical signaling channel we observed for the time interval between earliest streaming and the onset of collective pulsing with our droplet preparations (but not our membrane preparation) suggests that continuous streams and their fragmented versions (squads) can communicate via long range chemical signaling and not just by adhesive contacts. An improved theoretical approach that takes into account the branched structure of the streams and cell traction on different surfaces might well bring more understanding than the simple model we presented here which is incapable of predicting the apparent effect of the thickness of the signaling channel or its dependence on the nature of the substrate. Finally, we recognized the ubiquity with which many-cell collections (squads) which move as a single unit appeared throughout these preparations. Despite their universal appearance, we did not find them providing a means of forming aggregates by contact adhesion as the alternative pathway to aggregation through long range chemical signaling between isolated cells in sparse systems we expected. Asking whether we can understand the nature of these small scale life forms through cellular automata theory[45] one immediately acknowledges that while mobile multicellular structures are naturally predicted by such theories we are concerned that squads possess additional vital

capabilities for sensing and behavior that are beyond what current cellular automata theory can predict. Finally as a means of embracing all the phenomena we have focused on here: the synchronization of individual cellular emission through the diffusion of signaling molecules, and the coalescence of cells into squads and streams we propose that the discrete cell, chemically coupled oscillator approach of Nagano[46] be extended to include confined signaling channel effects. It would be exciting to see what such a theory would predict for the observations presented here, including fluctuations, since it could be a vital improvement over our rough theory.
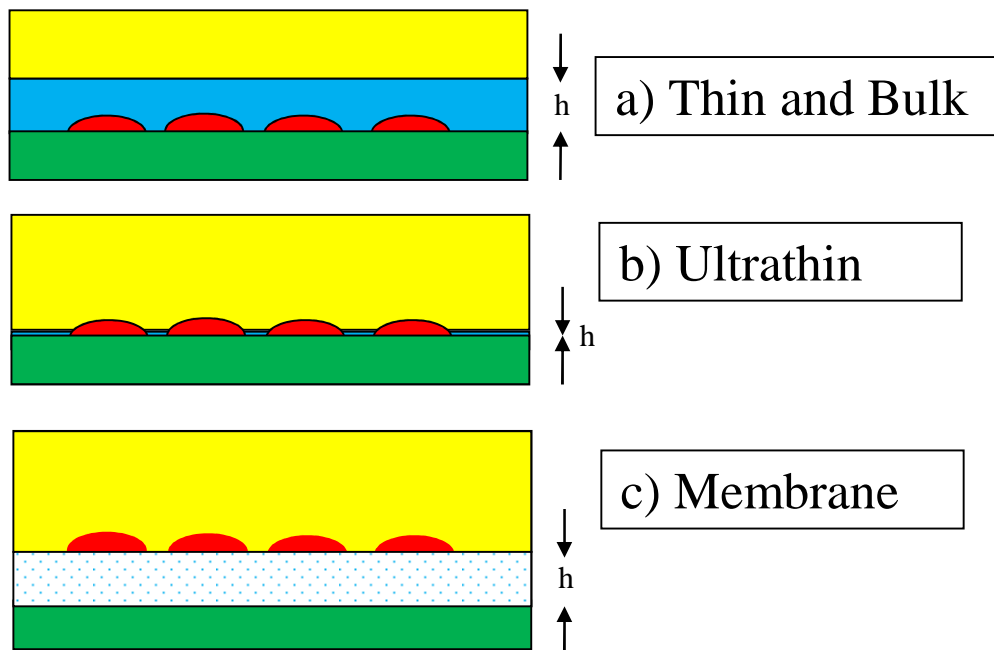
Acknowledgments

Figure 1: Various Schemes Used for Vertically Confining Aqueous Signaling Channels (whose thicknesses are indicated by h) in Side View.

a) Thin Film (h = 16 to 42 microns) and Bulk (h > 2000 microns), b) Ultrathin Film (h < 1 micron) and c) Membrane (h = 30, 60 microns). Glass substrate is green, aqueous layer is blue or dotted blue for membrane, cells are red, and confining mineral oil rich overlayer is yellow.
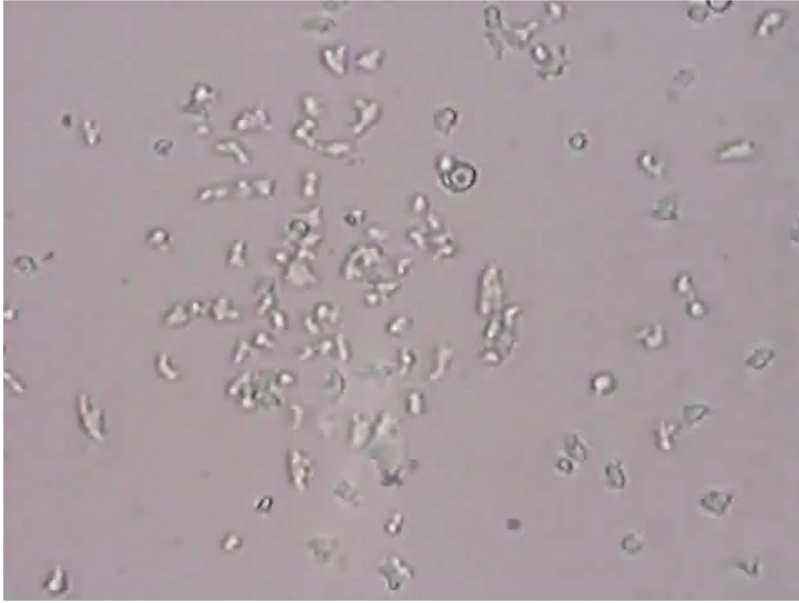
Figure 2: View of Dicty Colony in a 40 micron Thick Film Before Streaming.

The field of view is approximately 900 microns wide by 700 microns tall.

Figure 3: Squad (indicated by arrow) Growing by Accreting Cells. Field of view dimensions as in Figure 2.
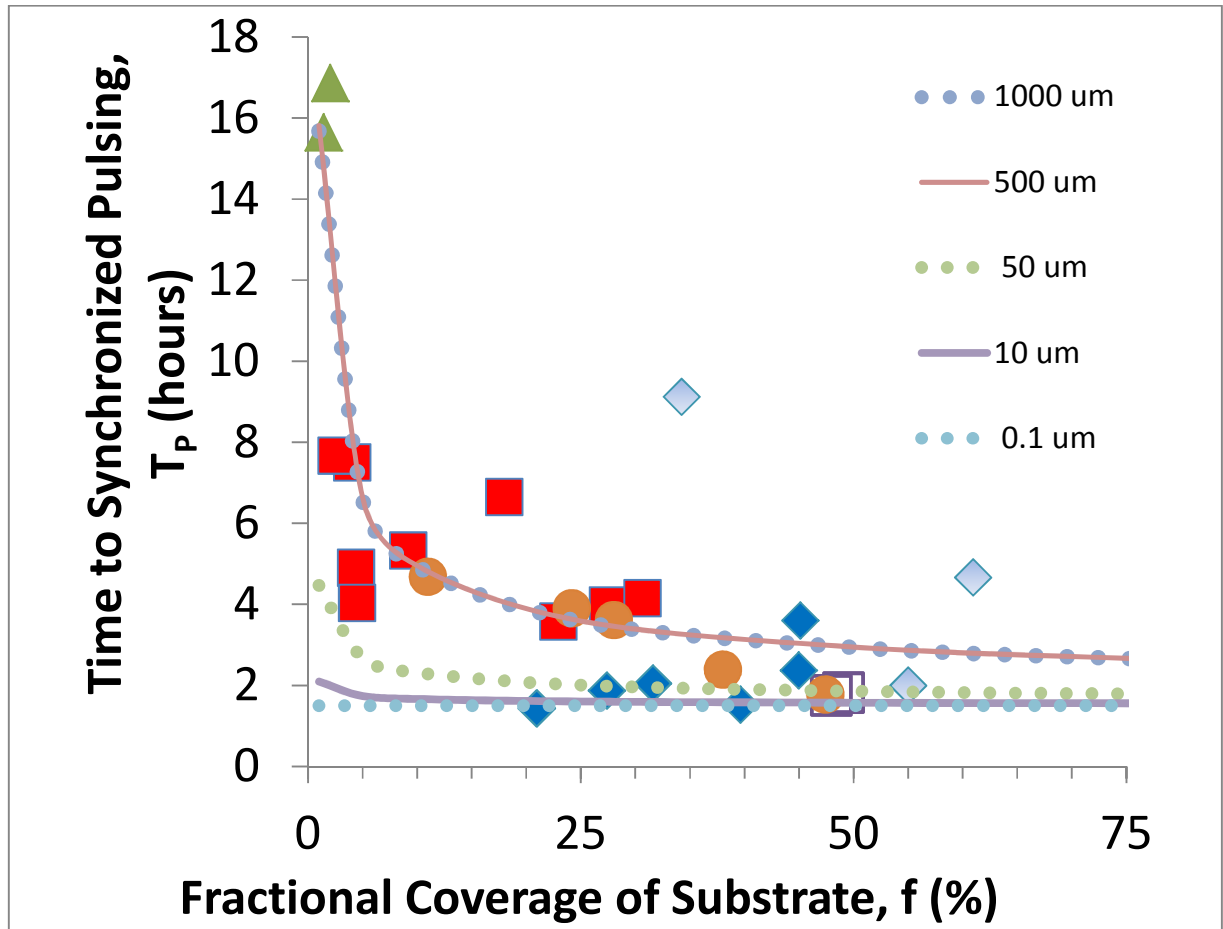
Figure 4: Time to Start of Synchronized Pulsation (T_P) **vs. Fractional Areal Coverage** (f) **for Bulk (squares), Thin (16 to 42 microns with most specimens in the 20 to 30 microns thick range, circles) and Ultrathin (less than 1 micron thick, diamonds, light coloring indicates that protocol followed deviated as described in text) Signaling Layers (open squares indicate that bulk synchronized pulsing had already begun by indicated time, triangles indicate duration of runs during which no synchronized pulsing was observed for bulk). As discussed in the text the uncertainty in establishing** T_P **for any given run is insignificant compared to the run to run scatter seen here. Our expected uncertainty in** f **based on repeated**

measurements for the same image are also insignificant. A notable feature is the low value for T_P for the thinnest films at intermediate coverage. The curves are synchronization theory (layer thicknesses indicated in legend, note that 500 and 1000 micron curves are indistinguishable) based on a fit development time of 1.5 hour and a fitted value (1.0 hour) for the time to synchronization for bulk layers at full surface coverage.
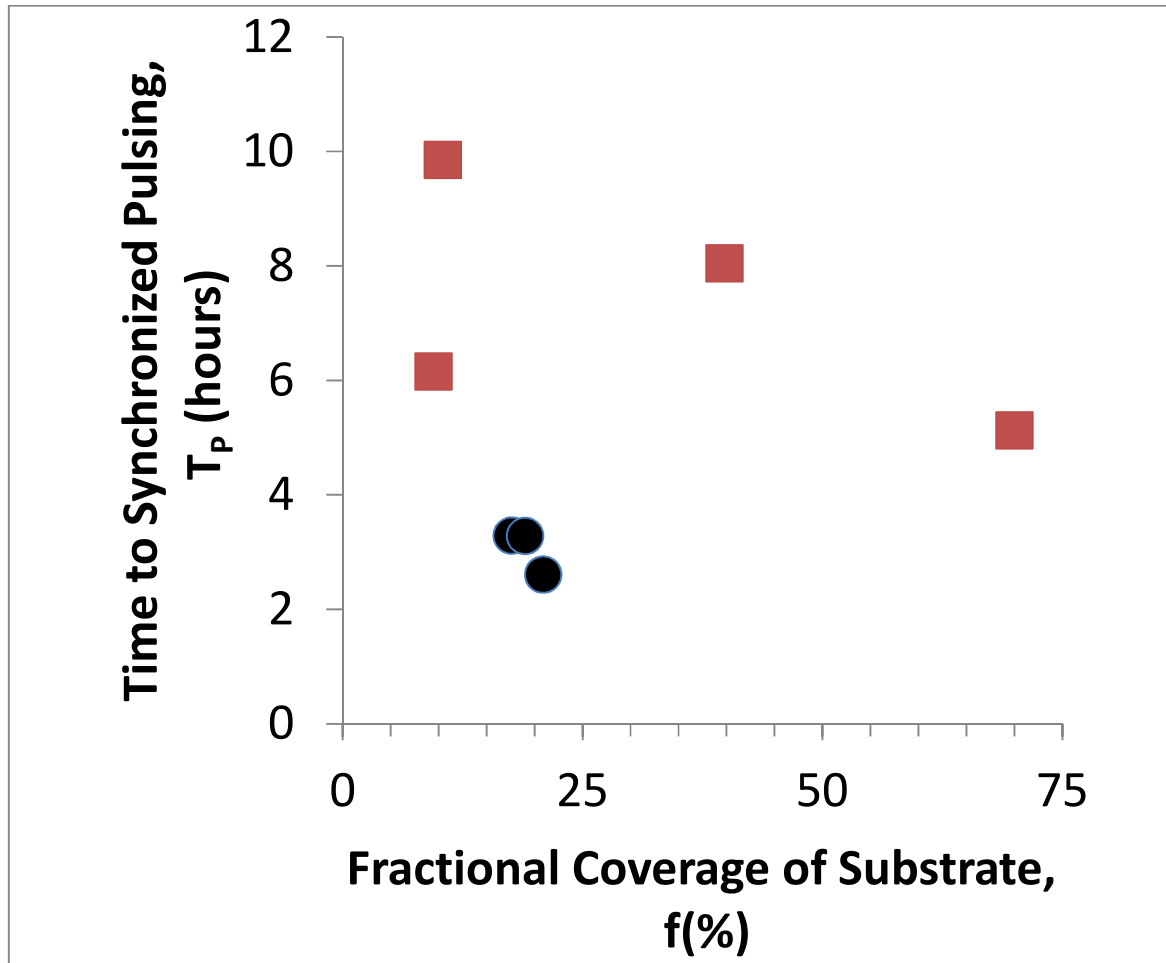
Figure 5: Time to Start of Synchronized Pulsation (T_P) **vs. Fractional Areal Coverage** (f) **for One (30 microns, circles) and Two (60 microns, squares) membrane preparations. Note the unexpected drop in** T_P **as the number of membranes is increased. Uncertainties as in Fig. 4.**
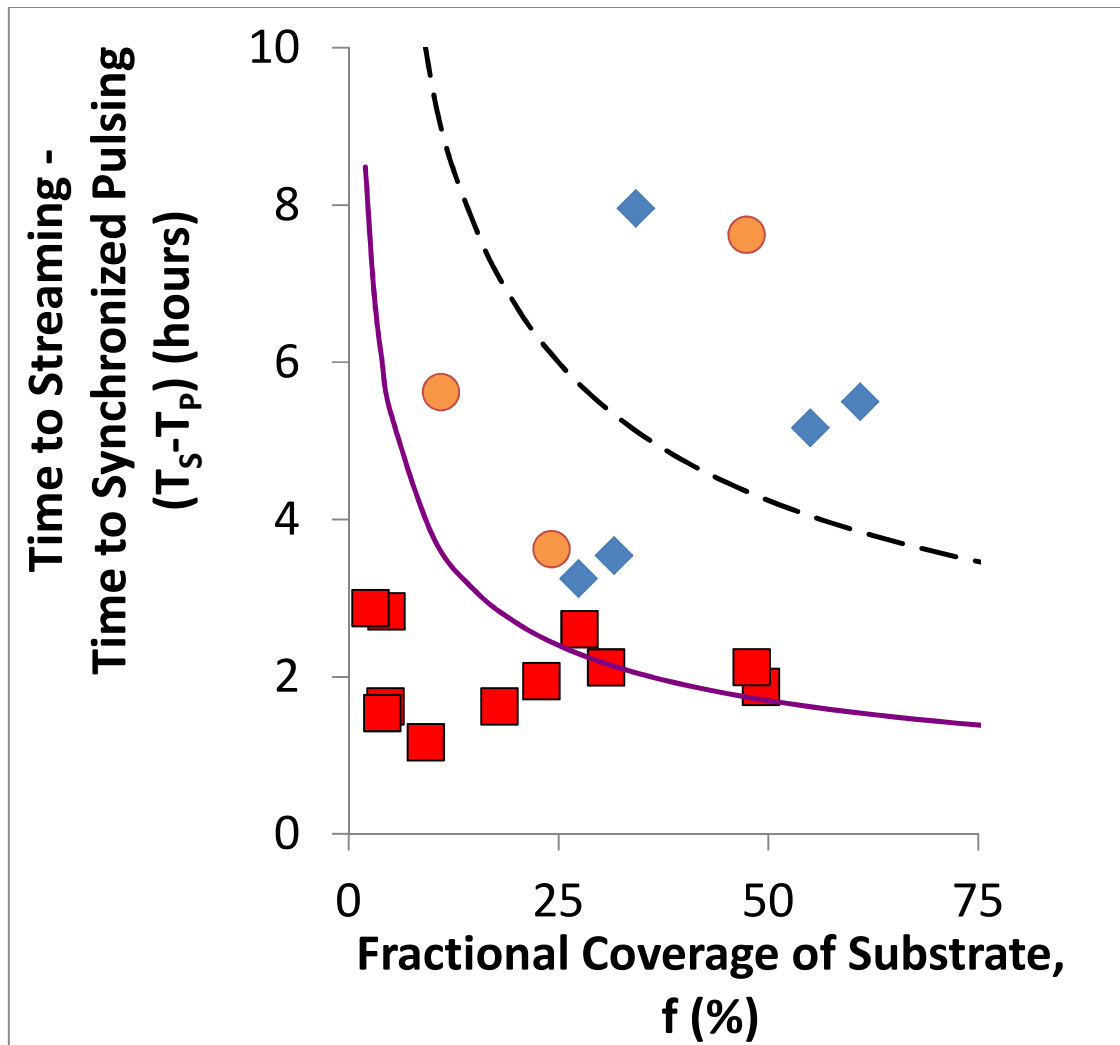
Figure 6: Interval between Start of Synchronized Pulsation and the Start of Streaming $(T_S - T_P)$ vs. Fractional Areal Coverage (f) for Bulk (squares), Thin Films (circles) and Ultrathin Films (diamonds). Uncertainty in the time interval based on repeated observations of the same video are estimated to be 0.2 hour, much less than the observed scatter in measurements. Expected relative uncertainty in f as discussed in the text is 0.06, which is insignificant for the work plotted here.

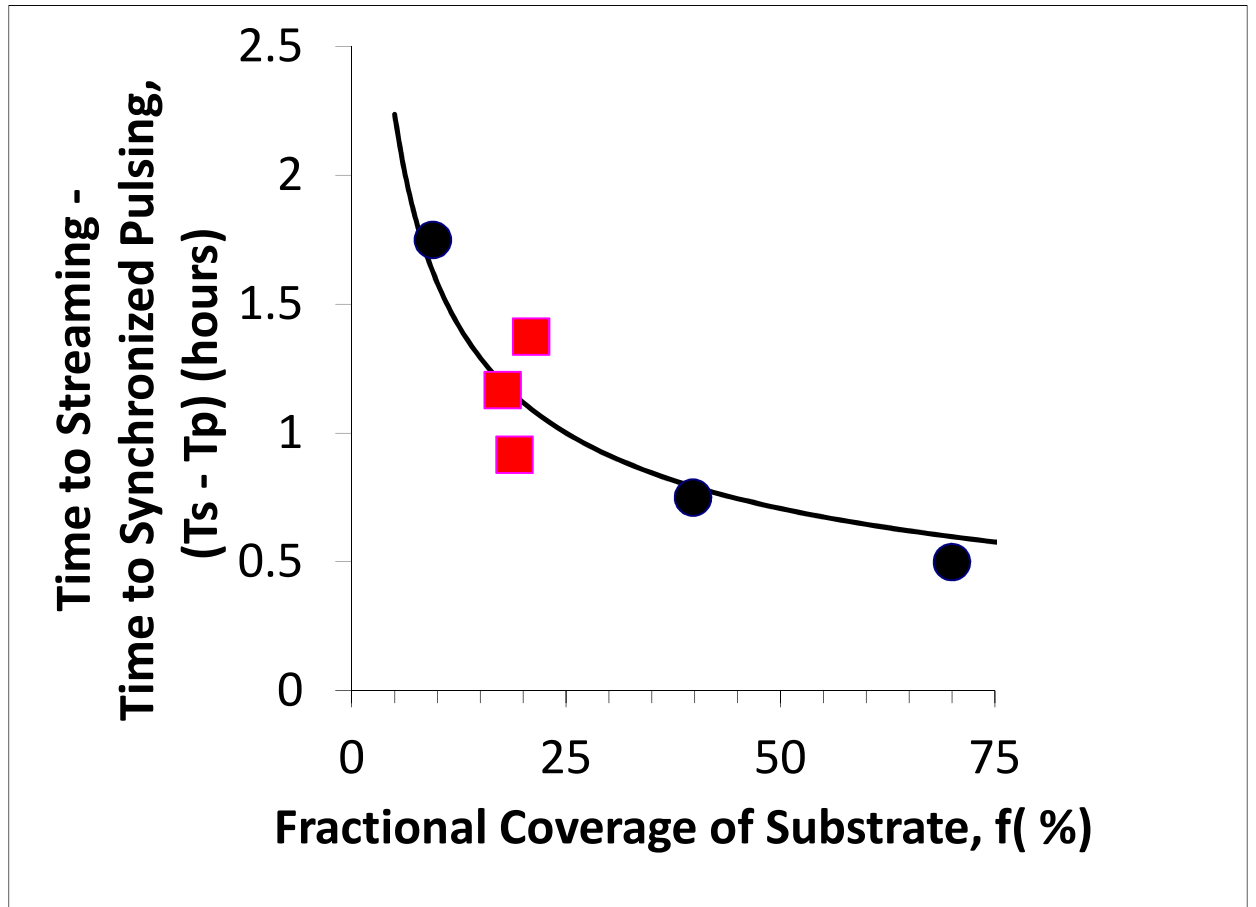**Sweep theory predictions as described in text plotted as curves.**

Figure 7: Interval between Start of Synchronized Pulsation and Start of Streaming $(T_S - T_P)$ **vs. Fractional Areal Coverage (** f **) for Membrane Preparations (30 microns thick, circles and 60 microns thick, squares). Sweep Theory prediction is given by curve. Uncertainties are as discussed in Figure 6.**

[1] Richard H Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), p. 27.

[2] For example, Satoshi Sawai, Peter A. Thomason, and Edward C. Cox, Nature **433**, 323 (2005).

[3] Gunther Gerisch and Benno Hess, Proc. Natl. Acad. Sci. (U.S.A.) **71**, 2118 (1974)

[4] Richard H Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), p. 102.

[5] Richard H Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), p. 103.

[6] Efforts at perturbing the chemical signaling system in the Dicty system have included the variation of cell density in all of the following experiments as well as: the application of cAMP that served to reset wave patterns by Kyoung J. Lee, Raymond E. Goldstein, and Edward C. Cox, Phys. Rev. Lett. 87, 0681011 (2001); fluid mixing by Colin McCann, Paul Kriebel, Carole Parent, Erin Rericha, and Wolfgang Losert, Amer. Phys. Soc. March 2007, Abstract U35.5; and the variation of the thickness of the signaling layer discussed here: Carl Franck and Kayvon Daie, Bull. Amer. Phys. Soc. March 2009, Abstract P39.6 and Carl Franck, Ryan Monaghan, Albert Bae, Duane Loh, and Eberhard Bodenschatz, Bull. Amer. Phys. Soc. March 2007, Abstract D34.9.

[7] Wolfgang Losert, Carole Parent, and Colin McCann, Bull. Amer. Phys. Soc. March 2009, Abstract W39.6; Colin McCann, Paul Kriebel, Carole Parent, and Wolfgang

Losert, Bull. Amer. Phys. Soc. March 2008, Abstract Q16.6; Colin McCann, Paul Kriebel, Carole Parent, Erin Rericha, and Wolfgang Losert, Amer. Phys. Soc. March 2007 Abstract U35.5; and Carl Franck, Ryan Monaghan, Albert Bae, Duane Loh, and Eberhard Bodenschatz, Bull. Amer. Phys. Soc. March 2007 Abstract D34.9 .

[8] http://www.dictybase.org/techniques/index.html .

[9] We used this particular strain with a gfp fusion protein containing plasmid (Wf38) as a convenience in our work since it was in use in our lab for other experiments involving fluorescence reporting at the time. We feel that the presence of the plasmid was irrelevant to the current discussion.

[10] http://www.dictybase.org/techniques/index.html .

[11] The substrates were subjected to radio frequency excited glow discharge in a reduced pressure air atmosphere for 30 to 60 sec. with a model PDC-32G Plasma cleaner/sterilizer, Harrick Plasma, Ithaca, NY.

[12] Stock number P50G-1.5-14-F, MatTek Corp., Ashland, MA.

[13] We used DB saturated mineral oil since we were concerned that if we had used pure mineral oil, the oil overlayer would upset the osmolarity of the aqueous layer in which the cells resided. We obtained the DB-saturated mineral oil from a phase separated liquid mixture of mineral oil and DB.

[14] Stock number 0121-1 light mineral oil, Fisher Chemical, Pittsburgh, PA.

[15] These quoted ranges include our uncertainty in establishing the thickness of each particular specimen, which is approximately 5 microns.

[16] We sometimes introduced a small clump of ground-up activated charcoal onto the substrate in order to collect Dicty waste products.

[17] One can expect monolayer coverage by adsorbed water on a glass surface underneath bulk DB saturated mineral oil (see similar situations in Carl Franck ``Wetting Experiments,'' in *Fundamentals of Inhomogeneous Fluids*, Douglas Henderson, ed. (Marcel Dekker, New York, 1992) p. 277). This is the liquid mixture analog of an adsorbed water layer on a hydrophilic substrate exposed to saturated water vapor.

[18] J. T. Bonner, Proc. Natl. Acad. Sci. (U.S.A.) **95**, 9355 (1998).

[19] Richard H Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), p. 5.

[20] Stock number JVWP02500 Millipore Corp., Billerica, MA.

[21] Hideo Urushihara, ``Cultivation, Spore Production and Mating,'' in *Dictyostelium discoideum Protocols,* Ludwig Eichhinger and Francisco Rivero, eds. (Humana Press, Totowa, NJ, 2006) p. 118. .

[22] Olympus IMT2 and IX71 brightfield microscopes using a 2.5x objective and CCD imaging devices.

[23] For example, Satoshi Sawai, Peter A. Thomason, and Edward C. Cox, Nature **433**, 323 (2005).

[24] Nikon Optiphot brightfield microscope with a 4x objective and a CCD imaging device.

[25] ImageJ by Wayne Rasband, National Institutes of Health, USA. http://rsb.info.nih.gov/ij/ .

[26] Deenadayalan Bakthavatsalam, Derrick Brazill, Richard H. Gomer, Ludwig Eichinger, Francisco Rivero, Angelika A. Noegel, Current Biology **17**, 892 (2007).

[27] For example, Herbert Levine and William Reynolds, Phys. Rev. Lett. **66**, 2400 (1991).

[28] Richard H Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), p. 102.

[29] The extracellular medium is not flooded with signaling molecules because cells excrete an enzyme, cAMP phosphodiesterase that served to degrade cAMP. We assume here that this enzyme is uniformly distributed. Richard H Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), p. 99. Clearly a more detailed description than we offer here would explicitly take into account its role in signaling dynamics.

[30] Nagano has stressed the importance of cell synchronization as the primary means by which Dicty establish communication links. See Seido Nagano, Phys. Rev. Lett. **80**, 4826 (1998).

[31] As has been introduced, for example in the cellular automata model of David A. Kessler and Herbert Levine, Phys. Rev. E **48**, 4801 (1993).

[32] By summing phasors to yield a Brownian walk.

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[35] Jason M. Brown and Richard A. Firtel, Trends in Genetics **16**, 191 (2000).

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[38] Martin Dworkin and Kenneth H. Keller, J. Biol. Chem. **252,** 864 (1977).

[39] Richard H. Kessin, *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity* (Cambridge University Press, New York, 2001), plate 1.

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[43] B. Wang and A. Kupsa, Science **277**, 251 (1997).

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[45] David A. Kessler and Herbert Levine, Phys. Rev. E **48**, 4801 (1993).

[46] Seido Nagano, Phys. Rev. Lett. **80**, 4826 (1998).