Hyperstructures

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Abstract

New concepts may prove necessary to profit from the avalanche of sequence data on the genome, transcriptome and proteome and to relate this information to cell physiology. Here, we focus on the concept of hyperstructures in which a variety of types of molecules are brought together to perform a function. The processes responsible for hyperstructure formation include changes in enzyme affinities due to metabolite-induction, transertion, and elevated local concentrations of proteins and their binding sites on DNA and RNA. We review the evidence for the existence of hyperstructures responsible for the initiation of DNA replication, the sequestration of newly replicated origins of replication and for cell division. We interpret cell cycle progression in terms of hyperstructure dynamics. Experimental techniques exist that can be used to study hyperstructures and we review some of the ones less familiar to biologists. Finally, we speculate on how a variety of *in silico* approaches could be combined to develop new concepts in the form of an *Integrated* or *Imaginary* cell – *I-cell* – which would undergo selection for growth and survival in a world of artificial microbiology.

1 Introduction

Molecular biology and biochemistry have provided a wealth of information about how RNA polymerases transcribe DNA into RNA and how ribosomes then translate mRNA into proteins, about the nature of those proteins and lipids that form membranes, and about other important molecules such as polyamines, polyphosphates and poly-β-hydroxybutyrate. Model organisms such as the bacterium *Escherichia coli* are invaluable in making sense of this information. The 4.6 Mb genome of *E. coli* K-12 MG1655 has been sequenced (Blattner *et al*., 1997) and was found to have 4288 protein-coding genes of which 38% had no attributed function. Other strains of *E. coli* have genes that range in number from 4085 (K-12 W3110) to 5361 (O157 RIMD) whilst the bacterium *Streptomyces coelicolor* has 7846. These numbers are in the same range of those of unicellular eukaryotes such as the fission yeast, *Schizosaccharomyces pombe*, with 4824 genes and the budding yeast, *Saccharomyces cerevisiae*, with 5885. But even when all genes are ascribed functions, how are we to interpret this information and use it to predict phenotypes? The challenge is to understand how cells organise their myriad constituents and processes. To explain how the concept of hyperstructures may help us, here we briefly review the bacterial cell cycle, focussing on the problem of division, and then discuss hyperstructures. We do this in the light of different questions: Why might a hyperstructure language be useful? What are hyperstructures? How do they form? How do they interact? How might they guide cells through state space to control growth, adaptation, differentiation and the cell cycle? We then discuss how the hyperstructure concept may help in exploiting the information provided by genome sequencing and how it may be tested using a combination of cellular automata and multi-agent systems. Finally, we advocate an approach to the study of biological complexity *via* construction of cell *in silico* based on hyperstructures. Such a cell would be an important step towards the construction of an integrated cell, *I-cell*, that would bring together many of the processes believed to determine the structure and phenotype of real cells.

2 The bacterial cell cycle

The principal events in the bacterial cell cycle include:

- *Initiation of chromosome replication from a single origin of replication*
- *The sequestration of newly replicated origins of replication*
- *Chromosome separation*

• *Chromosome segregation*
• *Cell division*
• *Inactivation of the division site*

In the case of cell division, it is still not clear how this event is timed, positioned and coupled to other events. The earliest known protein to act in *E. coli* is the tubulin-like FtsZ which migrates from the cytoplasm to a mid-cell location on the membrane where it assembles into a ring-like structure and where it recruits other division proteins. What lies upstream of FtsZ? Is it yet another protein or is it something else? We have shown that FtsZ can interact directly with phospholipid membranes in the absence of other proteins (Alexandre *et al* ., 2002). This is consistent with a major role for membrane dynamics in the regulation of the cell cycle as is the finding that membrane domains around the chromosomes differ from the domain at the future site of division (Fishov and Woldringh, 1999), this latter presumably being related to the large domains of cardiolipin observed at the division sites and poles (Koppelman *et al* ., 2001; Mileykovskaya and Dowhan, 2000).

The constraints on a solution to the division problem for *E. coli* are that the division site must be:

• *in the right place – midcell – to give daughters of similar sizes*
• *between chromosomes – to avoid producing a DNA-less cell*
• *formed at the right time in the cycle – perhaps to give the right DNA/mass ratio?*
• *formed at the right rate – to avoid, for example, cells getting bigger and bigger*
• *of the right nature – to allow membranes to curve and fuse whilst controlling ion and lipid fluxes*

It is in the context of trying to find a solution to this problem that we present hyperstructures.

3. Why invoke a hyperstructure language?

Cells survive and sometimes grow by somehow orchestrating millions of molecules of thousands of types to adapt to the environment and to proceed through the cell cycle. This entails cells solving the combinatorial problem of negotiating the immensity of state space since if each gene in *E. coli* were in either an on (transcribed) or an off (untranscribed) state, there would be 2^{4000} or 10^{1200} on-off patterns of gene expression (Kauffman, 1996). But there is more than this, there is also the epigenetic trap – cells in a population should not all have the same phenotype (else, for example, a single catastrophe would be more likely to wipe them all out). Exploring state space effectively boils down to: How can cells be both efficient and robust? We argue that the answer is that cells rely on an intermediate level of organisation – *hyperstructures.*

4. What are hyperstructures?

The sort of hyperstructure that concerns us here is the non-equilibrium type which is a large, extended structure of diverse molecules – genes, mRNAs, proteins, ions, lipids – that depend on a flow of energy/material for their existence. These hyperstructures are assembled to serve a specific function and are disassembled when no longer functional (Figure 1).

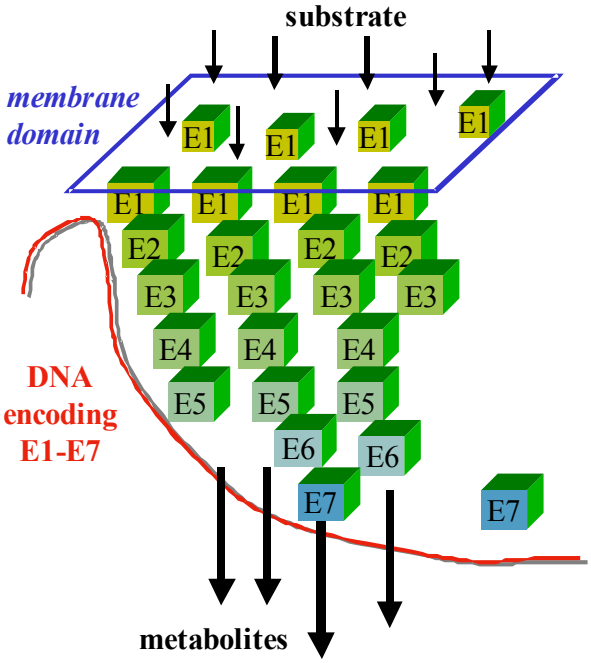

Figure 1: Formation of a non-equilibrium hyperstructure due to changes in the affinity of its constituent enzymes for one another. Enzymes E1 can only diffuse in the plane of the membrane whilst the other enzymes, E2 to E7 diffuse in the cytoplasm. The binding of a substrate, such as a sugar, to the E1 enzymes leads to an increase their affinity for one another and their assembly into an E1 domain. On binding its substrate, each enzyme in the pathway acquires an increased affinity for the following enzyme. This results in the assembly of metabolons E1 to E7 and the assembly of the hyperstructure (here, a group of metabolons). Note that transcription of the genes encoding E1 to E7 and the simultaneous translation of the mRNA may help the assembly of the hyperstructure (see plate 17).

They are essential for growth and for progression through the cell cycle. (There are also equilibrium or quasi-equilibrium hyperstructures that are not dependent on a flow of energy for their existence, that are important for survival in harsh conditions when growth is not possible and that can have an intimate relationship with non-equilibrium hyperstructures. They include condensed structures in chromosomes needed for bacteria to withstand irradiation (Wolf *et al.*, 1999) and to separate chromosomes (Bouligand and Norris, 2001).) The hyperstructure concept is relevant to the organisation of both prokaryotic and eukaryotic cells but here we confine ourselves to bacteria and in particular *E. coli*. The examples below are drawn primarily from the cell cycle and from metabolism. It should become clear that the archetypal bacterial hyperstructure contains genes being transcribed, their mRNAs being translated and the nascent proteins being inserted into the membrane to form a domain with a characteristic lipid composition (Figure 1).

Examples of possible non-equilibrium hyperstructures include:

4.1 Cell cycle.

The cell cycle comprises the initiation of DNA replication and the temporary sequestration of the newly replicated origins of replication (to prevent multiple rounds of replication), the separation and segregation of the chromosomes into the future daughter cells, and the division of the parental cell between the chromosomes, probably followed by the inactivation of the division apparatus.

An initiation/origin of replication hyperstructure. This comprises the DnaA protein and certain of the sites on DNA to which it binds. In *E. coli*, the ATP-bound form of DnaA is required for initiation of replication *in vitro* whilst the ADP-bound form is inactive (Castuma *et al*., 1993). Both ATP-DnaA and ADP-DnaA bind to 9mer DnaA boxes, TTA/TTNCACA, but only ATP-DnaA protein binds in addition to a 6mer site, AGATCT (Speck *et al*., 1999). DnaA is associated with the membrane *in vivo* (Newman and Crooke, 2000) and initiation requires a membrane with a particular phospholipid composition (Fralick and Lark, 1973) and domain structure to activate DnaA (Castuma *et al*., 1993; Xia and Dowhan, 1995) as well as the polymerisation of DnaA (Weigel *et al*., 1999). In one hypothesis, this putative hyperstructure has a transient existence that depends on the dynamics of other hyperstructures. It is clearly a non-equilibrium structure, indeed, it is disassembled after accomplishing the act of initiation (Norris *et al*., 2002a).

A DNA replication hyperstructure. This comprises the SeqA protein, key enzymes in DNA replication, and the genes that encode them (Norris *et al*., 2000). SeqA sequesters newly replicated origins and is found in clusters (Onogi *et al*., 1999). It binds to GATC sequences and it polymerises. The non-equilibrium nature of this hyperstructure results from its dependence on the energy-consuming process of replication with SeqA binding preferentially to hemi-methylated GATC sites in genes encoding enzymes responsible for DNA replication, topology, repair and precursor synthesis such as ribonucleoside diphosphate reductase (Guzman *et al*., 2002).

A cell division hyperstructure. This comprises the ten or so division proteins plus enzymes involved in peptidoglycan synthesis plus the genes that encode them (Buddelmeijer *et al*., 1998; Norris and Fishov, 2001). Many of these genes are located together and are transcribed together in the *dcw* cluster at the 2 min position on the chromosome. Recently, we have speculated that cell division may involve the conversion of lipid domains, positioned between the chromosomes, into tubes and vesicles in a dynamic, Golgi-like structure (Norris et al., in preparation). This is a non-equilibrium structure in that it requires (i) the energy-consuming processes of transcription and translation to bring these genes together at the membrane along with the nascent proteins (see below) and (ii) continuing and major changes in membrane structure.

4.2 Other hyperstructures.

These include a DNA compaction hyperstructure possibly involving the MukB protein which can form foci (Ohsumi *et al*., 2001); a nucleolus-like hyperstructure for ribosome synthesis (Lewis *et al*., 2000; Woldringh and Nanninga, 1985); a chemotaxis hyperstructure comprising chemotactic receptors such as Tsr with the kinase CheA and the transducing protein CheA (Bray *et al*., 1998; Stock and Levit, 2000) plus, we propose, the genes encoding the abundant chemotaxis proteins (and hence the necessity for hydrolysis of ATP and GTP during transcription and translation). Factors in the formation of possible hyperstructures for transport and glycolysis (Mitchell, 1996; Velot *et al*., 1997) are discussed below.

5. How do non-equilibrium hyperstructures form?

There are several complementary possibilities:

5.1 Metabolite-induction

• Non-equilibrium hyperstructures form when the cell is actively engaged in processing substrates and disappear when they are not

• These hyperstructures include enzymes in the same pathway and their genes
• Formation of certain of these hyperstructures may involve an interplay between diffusion in 2-D and 3-D in the sense that enzymes confined to domains in the 2-D membrane interact with other enzymes or groups of enzymes diffusing in the 3-D cytoplasm (Figure 1)

The evidence consistent with this scenario has been advanced for the existence of metabolons which are assemblies of the enzymes that act in succession in a pathway (Velot et al., 1997). Of course, such metabolons may themselves associate into larger hyperstructures.

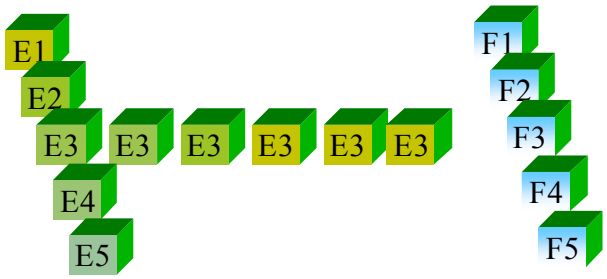

Figure 2: Horizontal links aid the assembly of a hyperstructure. Oligomeric protein E3 may bind together two identical metabolons (E1-E5 to E1-E5) or two different ones (E1-E5 to F1-F5). In the former case, E3 plays a role in the assembly of an individual hyperstructure whilst in the latter case E3 plays a role in the interaction between two different hyperstructures (see plate 18).

In the case of secretion, substrate binding promotes assembly of the 3 components of the ABC exporters of Gram negative bacteria e.g. in *Erwinia chrysanthemi* the substrate (protease) binds to PrtD (an ABC protein) which then binds to PrtE (membrane fusion protein) and which binds to PrtF (outer membrane protein) (Letoffe *et al*., 1996). In the case of glycolysis, the glycolytic pathway can be extracted as an equimolar complex of 1.65 MDa that reveals compartmentation of substrates (Mowbray and Moses, 1976). Evidence has also been obtained for the existence of metabolons which are assemblies of the enzymes that act in succession in a pathway (Mitchell, 1996; Velot *et al*., 1997). Of course, such metabolons, which may or may not be non-equilibrium structures, may themselves associate into larger structures or hyperstructures. In the case of import, sugar-specific phosphotransferase system permeases consist of EIIC and EIID in the membrane and EIIA and EIIB in the cytoplasm; EIIA is phosphorylated by HPr in a reaction catalysed by EI with P from phosphoenolpyruvate; E2s+E1+HPr are proposed to form a complex in response to the presence of the appropriate substrate as part of a 'metabolite-induced metabolon' event (Norris *et al*., 1999). An extension of this would be that a PTS hyperstructure forms when the cell is actively engaged in processing substrates and disappears when it is not. The idea here is that successive enzymes in the same pathway can be activated by their substrates to bind to one another in a heteropolymeric organisation that is sequential (or *vertical* insofar metabolic pathways are often drawn vertically with metabolites entering at the pathway at the top of the page and products as leaving at the bottom). A complementary idea is a single species of enzyme can be activated to oligomerize by substrate (Torshin, 1999); indeed, the full enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase and enolase – all glycolytic enzymes – results from their association. Again, this homopolymeric or *horizontal* organisation could help nucleate and stabilise hyperstructures (Figure 2).

5.2 Local concentrations

The phenomenon of oligomeric proteins binding to specific sites on DNA has been invoked to explain the operation of the *lac* and lambda repressors (Revet *et al*., 1999). It might also be invoked to explain the sequestering of newly replicated origins of replication by the protein

SeqA (Onogi *et al*., 1999) and the binding of DnaA to its sites. There are variations of this theme with, for example, the possibility that proteins such as the histone-like protein HU, which binds to both RNA and DNA (Balandina *et al*., 2001), could play important roles (see below) (Figure 3).

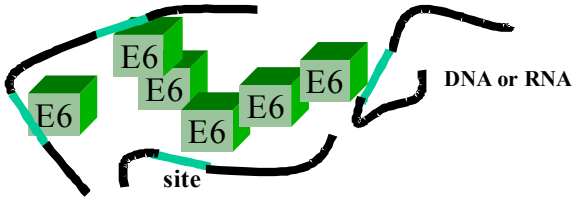

Figure 3: Local concentrations of oligomeric proteins can promote hyperstructure assembly. Protein E6 binds to its site (green) present in DNA or RNA to produce a region of the cytoplasm enriched in both E6 and its sites (see plate 19).

5.3 Transertion

Transertion is the coupled transcription, translation and insertion into and through membranes of proteins (Binenbaum *et al*., 1999; Lynch and Wang, 1993). The cytoplasmic membrane is composed of a wide variety of lipids and proteins so if these proteins have lipid affinities small proteolipid domains form.

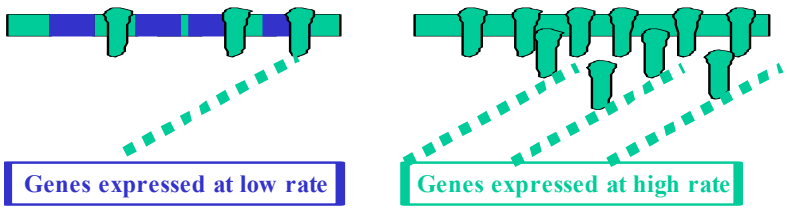

Figure 4: Transertion can nucleate hyperstructure assembly. Transertion, alias the coupled transcription, translation and insertion into and through membranes of proteins, may enrich a region of the membrane in the lipids (green) for which the proteins have an affinity. At a critical density of inserted nascent proteins, small proteolipid domains fuse into large ones and so nucleate hyperstructure assembly (see plate 20).

High rates of transertion may create a critical density of inserted nascent proteins that is sufficient for small proteolipid domains to fuse into large ones and so nucleate hyperstructure assembly (Norris, 1995) (Figure 4). For example, it might be supposed that the high density of transertion of the ATP synthetase components, which have lipid affinities (Arechaga *et al*., 2000; Ksenzenko and Brusilow, 1993), would result in assembly of an ATP synthesis hyperstructure.

5.4 Translated mRNA is protected from RNases and enzymes in metabolons are protected from proteases

It has been suggested that enzymes in complexes are more likely to escape proteolytic degradation than when not in complexes (Miller, 1996). An extension of this idea is that the partitioning of enzymes into a hyperstructure protects them from proteases – providing the latter are excluded from the hyperstructure. Hence an enzyme which has been assembled into a hyperstructure *because* of its activity is thereby preserved (i.e. active enzymes are preferentially protected). A similar argument is that mRNA translated within a hyperstructure could be preferentially protected from RNases on the outside of the hyperstructure.

6. How do hyperstructures interact?

6.1 Shared lipid affinities creates shared membrane domains

It can be argued that proteins with lipid preferences may congregate with those lipids in a positive feedback fashion to form the membrane domain part of a hyperstructure (see *5.3 Transertion*). Similarly, it might be expected that hyperstructures characterised by enrichment for a particular lipid would also tend to associate.

6.2 Shared binding proteins create shared cytoplasmic compartments

The idea is that certain abundant proteins may participate in the assembly of several different types of hyperstructures. This would enable a synergy whereby the progressive formation of a group of hyperstructures responsible for a set of functions would aid the recruitment of other related hyperstructures fulfilling complementary functions. Candidates for these proteins include IHF, FIS, and HU (for references see (Ussery *et al*., 2001)). IHF can modulate the transcriptional activity of promoters by influencing the looping of upstream DNA; the consensus site of IHF binding, YAACTTNTTGATTTW, lies within many repetitive extragenic palindromic sequences. FIS binding to upstream regions can enhance the transcription of highly expressed genes; the consensus for the FIS binding site is weak with estimates of its numbers ranging from 6 to 68000. HU binds to DNA with no evident sequence preference and, in so doing, influences the interaction of regulatory proteins with their specific sites on the DNA (Bonnefoy and Rouviere-Yaniv, 1992); HU also recognizes certain specific structures of both DNA and RNA with very high affinity and, for example, binds to the mRNA for RpoS (Balandina *et al*., 2001; Kamashev and Rouviere-Yaniv, 2000). In addition, there are over a 100 known activators and repressors of transcription in *E. coli* (Ouzounis *et al*., 1996) and it may be expected that these will control the synthesis of certain oligomeric proteins important in the assembly of different – but complementary – hyperstructures.

6.3 Gene distribution

There are strong compositional asymmetries in codon and amino acid usage depending on the orientation of the genes with respect to DNA replication and on the nature of the proteins encoded. This has led to predictions of different compartments for the syntheses of different proteins (Danchin and Henaut, 1997). A recent analysis of the distribution of genes in *E. coli* and *Bacillus subtilis* supports the hypothesis that one parental strand carries the set of genes required for growth in good conditions whilst the other strand carries those required for survival (resisting stress) in adverse ones; the idea is that one set of hyperstructures is associated with one strand whilst a different is associated with the other such that the segregation of these sets of hyperstructures underpins the segregation of the daughter chromosomes (Rocha *et al*., in preparation).

6.4 Water preferences

Water exists as nm-sized microdomains of differing structure and density that must have different solvent properties (Robinson *et al*., 1999; Wiggins, 1990). The difference in density appears to be of the order of 30% (Cho *et al*., 1997). Such differences should affect the distribution and activity of cellular constituents near, for example, the surfaces of membranes (Mayer and Hoppert, 1996). An important but difficult question is the extent to which the water preferences of the constituents of hyperstructures might determine hyperstructure formation and interaction.

6.5 Oscillations/vibrations

There are numerous oscillatory processes in eukaryotes of which the oscillation of protons and of NAD(P)H in neutrophils is particularly exciting (Petty and Kindzelskii, 2001). Such oscillations are candidates for playing global as opposed to local organising roles. Relating them to the dynamics of hyperstructures is a problem that has still to be addressed. In bacteria, several oscillatory systems have been discovered. For example, the Min system, which is involved in the selection or inactivation of the division site, oscillates with a periodicity of around 1 minute in *E. coli*; several explanations for these oscillations have been advanced (Meinhardt and de Boer, 2001; Norris *et al*., 2002b).

7. Cell division

The regulation of cell division can now be considered in terms of the dynamics of hyperstructures. It has been argued that one of the functions of the bacterial cell cycle is to generate daughter cells with different phenotypes since this would allow the population to both explore all the possibilities for growth offered by the environment and be ready for a sudden catastrophic change (Norris *et al*., 2002a; Segre *et al*., 2000). In this scenario, during the run-up to initiation, the mass to DNA ratio increases and certain hyperstructures become 'stronger' by attracting ever more of the cell's resources (such as the transcriptional and translational apparatus) whilst other hyperstructures are weakened and disappear (Norris *et al*., 2002a) (Figure 5). This results in a drop in the diversity of hyperstructures, some of which release DnaA as they dissociate, a DnaA-initiation hyperstructure forms, and replication of the chromosome begins. Now suppose that short FtsZ polymers are associated with glycolytic and other hyperstructures so that FtsZ is effectively sequestered (noting that, at least in eukaryotes, tubulin is associated with glycolytic enzymes (Lloyd and Hardin, 1999)). This leads us to consider three complementary possibilities. One is that the FtsZ-sequestering hyperstructures are temporarily disrupted by chromosome replication to release FtsZ which can then participate in division. Another possibility is that the changing activity of the phosphotransferase system/glycolytic hyperstructure directly leads to its own disassembly (for example, its capacity might exceed demand and lead to feedback inhibition) and releases FtsZ. This would be consistent with the advance in divisions in synchronous cultures of *E. coli* induced by addition of the non-metabolisable, glucose analogue α-methylglucoside (Fishov, 1994) and the delay in divisions induced by transfer to a rich growth medium (Kepes and Kepes, 1985). Finally, FtsZ may exist as a delicate cytoskeleton that extends throughout the cell, as described for chloroplasts (Kiessling *et al*., 2000), that may act as a metabolic sensor leading to its redistribution in cell division.

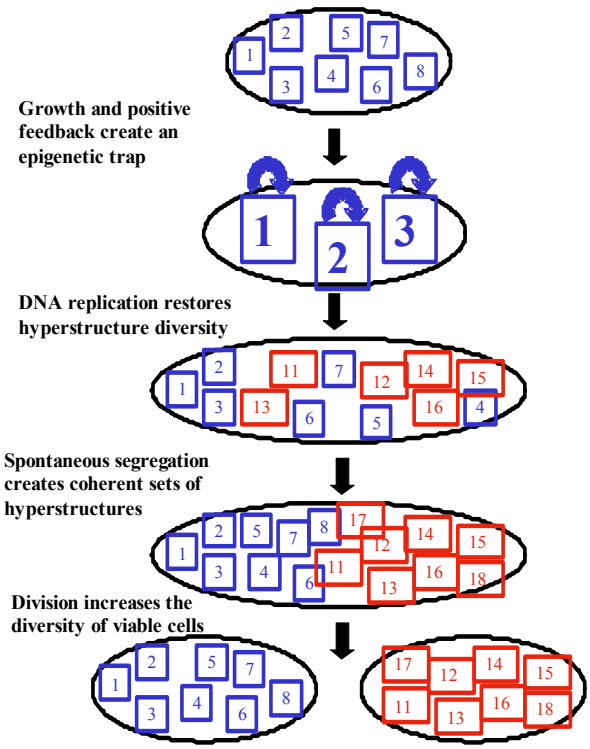

Figure 5: Cell cycle progress as a state cycle of hyperstructures. Rectangles represent non-equilibrium hyperstructures each performing one function. Blue rectangles correspond to hyperstructures with a common set of lipid (or other) preferences whilst red rectangles correspond to hyperstructures with a different set of preferences (see plate 21).

Before trying to put it all together, we should bear in mind that, all else being equal, the rates of transcription of two copies of the same gene diverge if this gene is –vely regulated *in trans* but +vely *in cis* (Norris and Madsen, 1995). The –ve regulation *in trans* could result from a repressor diffusing through the cytoplasm to each separate stretch of DNA whilst the +ve regulation *in cis* could result from an RNA polymerase transcribing a gene making this particular stretch of DNA accessible to another polymerase. This leads to the conclusion, surprising for many microbiologists, that two identical chromosomes in the same cytoplasm (which contain many such genes) *therefore* have different patterns of gene expression. The same argument can be made in terms of hyperstructures: a set of genes is expressed from one chromosome to form part of a hyperstructure; this assembly involves positive feedback between the constituent ions, lipids, proteins and nucleic acids since as the density of one constituent in a region increases, the probability increases that the density of another constituent will also increase. In the context of a cell in which hyperstructures compete for existence (that is, negative regulation *in trans*), the result is a highly structured, asymmetric cell in which each future daughter cell has a different set of hyperstructures associated with it and these sets differ in their composition of lipids, ions, water structures, proteins, mRNA and expressed genes (Figure 6). At present, it is difficult to discriminate between the different ways in which the principal proteolipid domains around the chromosomes could create a division site (red, dotted arrows in Figure 6). In one scenario, the site would simply consist of the *interface* between the two domains whilst in the other scenario, the site would consist of a distinct *domain* between the principle two domains. There are, of course, permutations of these possibilities.

The essence of our proposal is that hyperstructure dynamics could achieve:

• separation of chromosomes during replication

• differentiation of both chromosomes and membrane
• the right place for a site to attract and activate division enzymes (between the chromosomes)
• the right time for the creation of a division site (after chromosome segregation)
• the right nature for a division site - a potential non-bilayer
• coupling between replication, segregation and cell division
• a calcium flux (down the concentration gradient)
• orchestration of membrane-activated kinases, proteases *etc.*

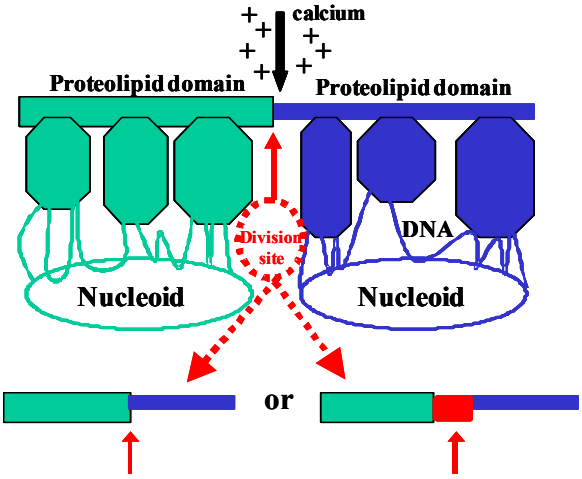

Figure 6: The spatiotemporal control of cell division by hyperstructures. The hyperstructures (the green or blue polygons) form one of two sets depending on the common preference within a set for lipids, ions, proteins etc. Each set is associated with a chromosome and is present in the future daughter cell. The division site is in the cytoplasmic membrane (thin rectangles) at the interface between these sets indicated by the arrow. Two possibilities for the structure of the division site (red arrows) which may be between the principal domains (blue and green) at either the interface or a separate, specific domain (red) (see plate 22).

8. The advantage of organisation at the level of hyperstructures

It has been observed that the difficulty of administering a laboratory is proportional to the square of the number of members of the laboratory, N^2 (Bok, 1983). This difficulty, D, is reduced if the individuals are put into N_1 groups such that D equals the square of the number of groups (to reflect group interactions) plus the square of the number of individuals in each group N_0^2 (to reflect interactions within groups) times the number of groups:

$$D = N_1^2+(N_0^2)N_1$$

Hence

$$D = (N/N_0)^2+(N_0^2)N/N_0$$

And

$$D = N^2/N_0^2+N_0N$$

To minimise D,

$$\delta D/\delta N_0 = -2N^2/N_0^3+N = 0$$

Hence the difficulty is at a minimum when

$$N_0 = (2N)^{1/3}$$

This formula helps to give us a feel for the numbers of hyperstructures that may exist in a cell. Just considering proteins, for example, a bacterium containing of the order of a million interacting proteins would be expected to have around a hundred hyperstructures. The existence of this intermediate level of organisation therefore means that the problem of generating a

limited number of coherent phenotypes that are adapted to survival and/or growth is greatly simplified. Navigation through the immensity of state space becomes a choice between 100 or so hyperstructures rather than 4000 plus genes – 2^{100} on-off combinations rather than 2^{4000}. To generate a coherent phenotype, for example, enzymes appropriate for growth in cold oxygenated conditions should not be synthesized in the same cell at the same time as those for growth in hot anaerobic conditions. Coherence can be achieved because cells can manage the relatively few common factors required to bring together a particular set of hyperstructures. The existence of hyperstructures also allows, we speculate, bacterial cells to regulate DNA replication and cell division so as to create heterogeneous populations that can both grow and survive unexpected challenges.

9. Using the hyperstructure concept to exploit sequence data

Of the numerous *in silico* approaches possible, we focus here on cellular automata which are used to model many physical and biological phenomena (Vichniac, 1984). Once the units that constitute the automata have been assigned initial states, the evolution of these states can then depend on both the previous history of the state and on the state of neighboring units. Hence, cellular automata can be particularly suitable for modeling the dynamics of interactions between molecules in 3 dimensions. We now use cellular automata to illustrate how they might be used to model the effects on hyperstructure assembly of the following:

9.1 Metabolite-induction

To determine the values of the parameters governing the formation of hyperstructures in bacteria, we have constructed a preliminary version of a cellular automaton program (with features of multi-agent systems) that simulates the dynamics of the localization of the PTS and glycolytic enzymes in both a 2 dimensional membrane and a 3 dimensional cytoplasm (Le Sceller *et al*., 2000). Each unit volume represents a 10nm x 10nm x 10nm cube in a cell that can have a maximum volume of 200 x 200 x 200 unit volumes or $8\mu m^3$. This is more than sufficient to represent *E. coli* which in certain growth conditions has a volume of 2 cubic microns. Each cubic unit volume in the membrane is surrounded by 8 other unit volumes and each unit volume in the cytoplasm is surrounded by 26 others. At each time step, all enzymes are considered in a random order. Each can move into a free neighboring unit volume. In this preliminary study, there was a structuring of both membrane and adjacent cytoplasm and hyperstructures were generated containing up to 500 enzymes.

9.2 Transertion

To model the anchoring effect of transertion on nascent proteins (Figure 4), a proportion of the PTS Enzymes II (for example) could be permanently confined *in silico* to a patch of the membrane. An important parameter may therefore be the *area* over which these proteins are inserted. It is not easy to obtain this area experimentally with current techniques (but see the NanoSIMS below). However, this may be an instance when the simulation reveals whether hyperstructure formation is very sensitive to the area of transertion and therefore whether energy should be invested in performing the relevant experiments.

9.3 Lipid preferences

The cosegregation of proteins with the lipids for which they have pronounced affinities is a potent way to produce domains. This process may be simulated in the 'membrane' of cellular automata given these affinities. Below (*10.3*), we suggest a series of experiments that could

lead to consensus sequences for lipid binding and hence a way, ultimately, to convert sequence information into the 'lipidome' and facilitate the simulation of the distribution of all membrane proteins.

9.4 Local concentrations

Using cellular automata to model local concentrations might exploit knowledge of DNA-binding proteins and their sites providing DNA can also be introduced into the model. One way to achieve this would be to divide the chromosome into chunks comparable in size to proteins. Each chunk would be constrained in its diffusion by a function inversely proportional to the distance between the chunk in question and another chunk. It may also prove necessary to make efforts to model reptation, the constrained movement of polymers in a crowded solution.

9.5 DNA distribution

DNA curvature, flexibility and stability have been analysed for 18 fully sequenced bacterial genomes (Pedersen *et al*., 2000). This reveals many significant structural features including a set of 20 regions with identical and extreme structural properties that are proposed to function as topological domain boundaries. These features are presumably related to the properties of proteins such as HU (see *6.2*) which binds preferentially to unusual structures such as kinked or cruciform DNA (Bonnefoy *et al*., 1994; Kamashev and Rouviere-Yaniv, 2000). The challenge is to translate this information into a dynamic 3-D model taking into account that much of the DNA is probably in a cholesteric form. One model that might be tested *via* cellular automata (as in *9.4*) is that HU both binds to these curved regions and self-associates such that curved regions are stacked at the edges of twisted liquid crystalline regions. In such a model, the terminus region, which has high curvature, low flexibility and low helix stability (Pedersen *et al*., 2000), might be expected to exhibit a distinctive packing.

9.6 Parallel approaches

In an activity-based vision of the cell, only a subset of its constituents is important in determining the phenotype of the cell at any one time (Norris, 1998). This subset comprises those constituents that are *active* where *active* is considered to mean being transcribed for a gene, being translated for a mRNA, and catalysing a reaction for an enzyme. Belonging to this active subset requires a competition between constituents that were active in the previous time period (the *status quo* factor) and constituents that act in synergy with one another (the coherence factor).

In this section, we describe a new implementation of cellular automata or units based on the related idea that only a few unit volumes are potentially active, that is either contain a molecule or a have a neighbouring unit containing a molecule. The advantage is that memory is not needed to store these empty units. This leads to a time and memory efficient approach for computing the successive generations of the units. The overall state of the system is determined by the content of all the units at a given time. Computing the next generation means determining the new state of the system after the application of all the local rules to each unit. This process must not depend on the order the units are examined and, ideally, each unit is treated independently of all the other units. The standard way to represent the 3-D space is to use a array of structures to address each unit that often contains only a number. Using this method, it is easy to determine the neighbourhood of a unit by a simple transformation of its coordinates, and then access the array to get the values of the neighbouring units. The major drawback is that we must store *all* the units, even the empty ones.

In our approach, we also represent the space by a three coordinate system, but we store in the computer memory *only* the active or potentially active units (i.e. those that are filled or next to filled units). This reduces the memory cost and allows us either to reduce the size of each unit to have a more accurate simulation, or to simulate a larger space. The potentially active cells are stored in a hash table which allows a very fast access time, comparable to the access time of a 3-D array, if a good hash function is used along with an adapted strategy to resolve collisions. This low cost implementation of the state of the system can be used to reduce the time used to compute each generation if an extra cost is paid by duplicating the representation of the space: the local rules are applied to each active unit using the values from the first, *current* space and the result is stored in the second, *new* space. After all the units of the first space have been processed and the second space is complete, the second space becomes the current space and the next generation can be computed.

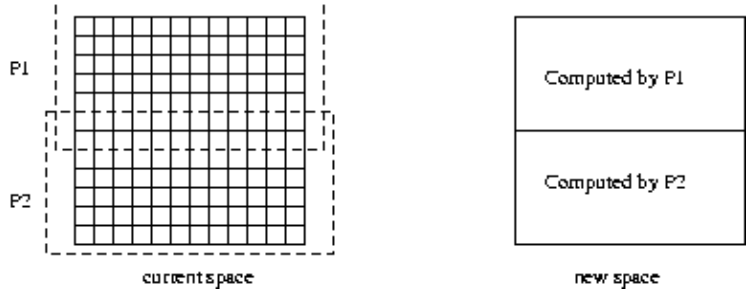

Figure 7: A two process example of parallel processing. Since each process does not need to access the space of the other one (except the boundary of the current space), each part can be stored locally in a multi-computer networked environment. The boundary of each part is the only information to be shared (i.e. transmitted between the computers).

Since the current space is only accessed for *reading* values whilst the new space is only accessed for *writing* results, the current space can be freely accessed by multiple processes without synchronisation. The new space can be split into parts that can be computed separately on a multiprocessor with a consequent dramatic reduction in computation time. Each process requires its own part of the current space but also acts on a surrounding layer of single units in the parts treated by other processes. Since each process only accesses the part of another process at the boundary, each part can be stored locally in a multi-computer networked environment.

In the 12 by 12, 2-D example (Figure 7), process P1 only needs to access the first 7 lines (0 to 6) of the current space to compute the first 6 lines of the new space, while process P2 needs to access the last 7 lines (5 to 11) of the current space to compute the other half of the new space. Since there is no read/write conflict between P1 and P2 no synchronization is needed. This is another advantage of the inherent parallelism of this implementation.

9.7 Hyperstructure movements and reactions

Interactions *between* hyperstructures are proposed to result in a pre-divisional cell with one set of hyperstructures in one half the cell and a different set in the other half. Such sets of hyperstructures may be formed on the basis of common lipids, ions, binding proteins and/or water properties. Movements of hyperstructures are nicely illustrated by the SeqA-replication hyperstructure that, during the cell cycle, goes from a single focus to two foci that then migrate to the one-quarter and three-quarter positions (Ohsumi *et al*., 2001; Onogi *et al*., 1999). To model how interactions between hyperstructures might lead to redistribution of hyperstructures within the cell, we consider a cellular automaton model in which several hyperstructures can be

represented simultaneously in a coarse-grained way (so that the units are bigger than single macromolecules).

The idea presented in this section entails providing local rules to reproduce molecular reaction and diffusion using cellular automata. The difference between our approach and typical reaction-diffusion processes is that the molecule concentration (in a specific position) is boolean: *true* if there is a set of molecules, *false* if there are none. One of the simplest systems has only one type of molecule on a 2-D grid (environment). Focusing on a particular molecule and its neighbourhood, it is clear that a unit plus the 8 adjacent units is a square of side 3 units. If we suppose that the molecule can move or stay in the same place, the molecule will have 9 possible positions (Figure 8).

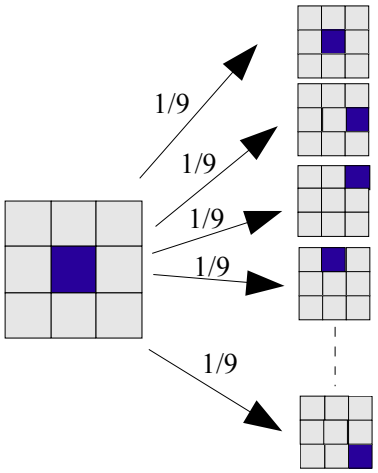

Figure 8: Movement on a 2-D grid. A molecule at time *t* can choose between 9 positions at time *t+1*. A molecule at time *t* can choose between 9 positions at time *t+1*.

With cellular automata, the state of a unit depends on its neighbourhood. Thus, a local rule must be used to determine whether a unit in the 2D-grid becomes *true* (has molecules) or *false* (is empty). The idea is to invert the arrow direction in the previous figure. Thus, if an empty cell is adjacent to one filled cell, it has a probability of $1/9^{th}$ to become filled. We can apply this to any neighborhood.

Given a unit in a 2D-grid, the probability of the unit to become *true* (filled) is $p = n / 9$ where n is the number of filled units into its neighbourhood. More generally, for a *dim*-dimensional environment (a grid of dimension *dim*), the probability of one cell to become *true* is:

$$p = n / N \qquad (1)$$

where $N = 3^{dim}$. Figure 9 shows the results of this local probabilistic rule (rule 1) with a *60x60* grid at three different times.

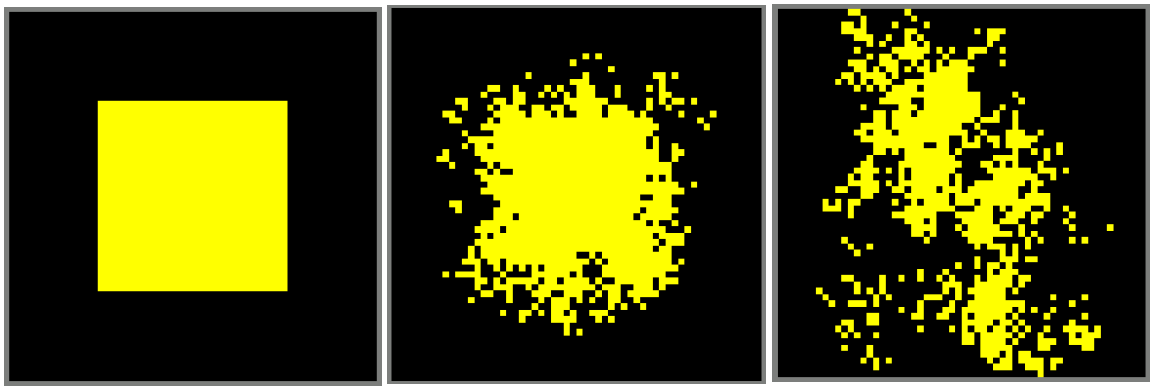

Figure 9: Diffusion in a cellular automata system. Empty units are black and filled units are yellow. States at successive times (t=0, 10 and 100) are shown.

To construct a multi-molecule hyperstructure, the next stage consists in putting together different types of molecules. In this case, the value of a unit is not a boolean but an integer included between *0* and *nb* (number of types of molecule). In this way, rule (1) becomes

$$\begin{aligned} p_0 &= n_0 / N \\ p_1 &= n_1 / N \\ p_2 &= n_2 / N \\ &\dots \\ p_{nb} &= n_{nb} / N \end{aligned} \qquad (2)$$

where n_i is the number of molecules of type i into the neighbourhood and $N = 3^{dim}$ into the *dim*-dimension grid. An empty cell is of type *0*.

To choose the future type of a cell among all the possibilities, we consider a real random number A bounded by *0* (included) and *1* (excluded). The decision rules are the following:

$$\begin{aligned} &\text{if } A \in [0, p_0[\text{ then the considered cell is of type } 0 \\ &\text{if } A \in [p_0, p_0+p_1[\text{ then the considered cell is of type } 1 \\ &\dots \\ &\text{if } A \in [p_0+p_1+\dots+p_{nb-1}, p_0+p_1+\dots+p_{nb}[\text{ then the considered cell is of type } nb. \end{aligned} \qquad (3)$$

Figure 10 shows the results of these local probabilistic rules with a *60x60* grid at three different times and for two types of molecule.

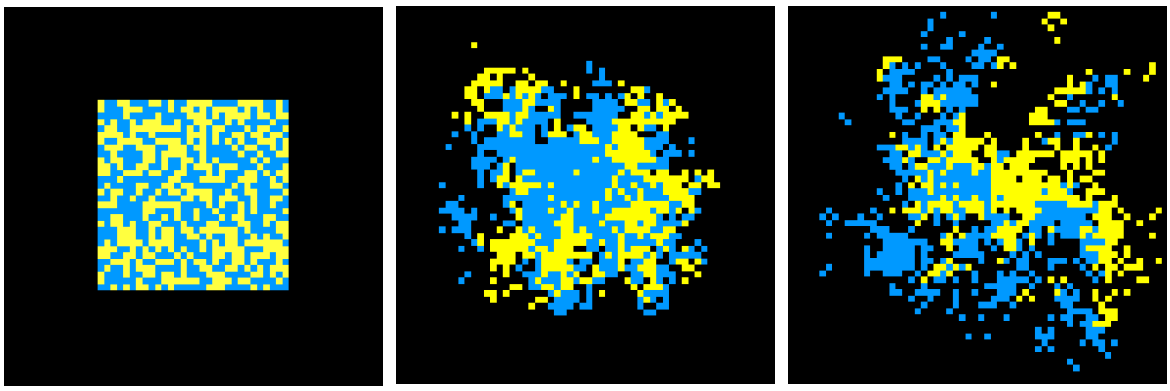

Figure 10: Diffusion of 2 types of molecule in a cellular automata system. States at successive times (t=0, 10 and 100) are shown (see plate 23).

To study hyperstructures containing many different molecules that can perform chemical reactions, we allow two different molecules to react to produce another type of molecule:

$$mol_i + mol_j \rightarrow mol_k \qquad (4)$$

To do this, we add the following simple rule (5) to rule (3):

if the *considered cell* is of type *i* (resp. *j*) and the type chosen thanks to rule 3 is *j* (resp. *i*) then the cell will have the type *k* (according to rule 4) (5)

Figure 11 shows the evolution of the system in a *60x60* grid at three different times, for two types of molecule and that react together according to rules (4) and (5) to produce a third type of molecule.

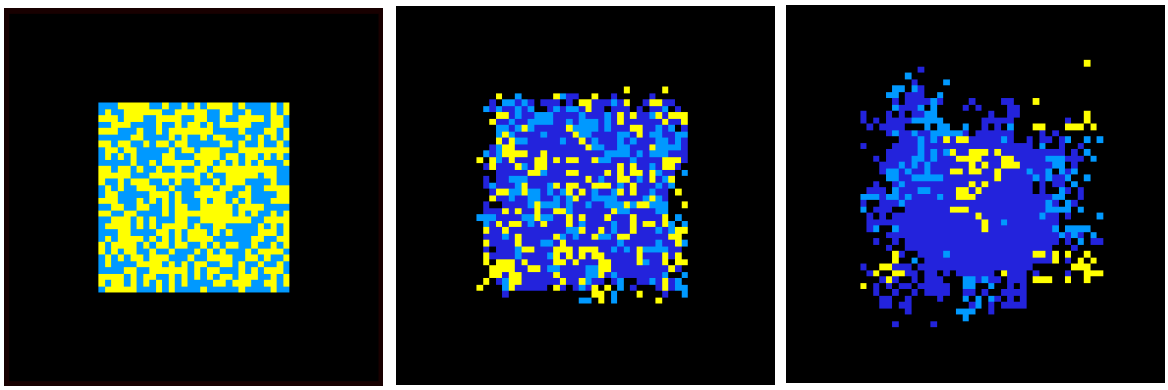

Figure 11: Diffusion of 2 substrates and a product in a cellular automata system. Molecules of type 1 and molecules of type 2 interact to produce type 3. At time t=0, there are only 2 types of molecules, type 1 (yellow) and type 2 (light blue). At time t=3, type 3 (dark blue) appears (see plate 24).

To observe the formation of and interaction between hyperstructures, we introduce the notion of affinity between molecules. In our example, molecules of type 1 are activated and can therefore bind one another. Molecules of type 2 and 3 have similar behaviours. Moreover, molecules of type 1 can react with molecules of type 2 to produce molecules of type 3.

- Molecules of type 1 come from the top of the cellular automata and bind together
- Molecules of type 2 come from the bottom of the cellular automata and bind together too
- Molecules of type 1 react with molecules of type 2 to produce molecules of type 3
- Molecules of type 3 bind together and with molecules of type 1 and type 2.

10 Experimental aspects

10.1 NanoSIMS

Visualising hyperstructures directly with conventional techniques has been difficult since it requires the co-localization of such disparate elements as proteins, mRNA, genes and lipids at the 50 nm scale. In secondary ion mass spectrometry, a section of biological material is subjected to a beam of ions that pulverizes it to release secondary ions that are filtered by mass spectrometry to allow an image to be obtained (Thellier *et al*., 1993). Recent developments in NanoSIMS technology are very promising since the new generation of machines provides

resolution at the scale required and allows detection of isotopically marked probes to proteins and nucleic acids. This opens up the exciting possibility of studying hyperstructures by imaging simultaneously both nucleic acids and up to 10 different proteins at a resolution intermediate between light and electron microscopy.

10.2 Optical waveguide lightmode spectroscopy (OWLS)

In the case of glycolysis, we lack details of the exact abundance of proteins such as phosphoglucose isomerase**,** fructose -1,6-P2 aldolase, triose-P isomerase, glyceraldehyde 3-phosphate dehydrogenase A complex, and phosphoglycerate kinase. Although we can obtain these *via* radioactive labeling and 2-D gel electrophoresis, there are attractive, recent techniques such as those based on isotope-coded affinity tags (Gygi *et al*., 1999). More seriously, we lack details of the constants of affinity of the PTS and glycolytic enzymes. These could be obtained using OWLS in experiments with purified proteins and substrates (Ramsden, 1993). By introducing and removing the substrates, it may also prove possible in these experiments to estimate the period of time for which an enzyme remains active (i.e. has a higher affinity constant) once its substrate has gone (Ricard *et al*., 1998).

10.3 MALDI-MS and ES-MS

We are presently using sensitive techniques of mass spectrometry to explore the possibility that concomitant with overproduction of a membrane protein is a compensatory overproduction of the lipid for which it has an affinity. If this approach is successful, a semi-automated, general strategy might be developed in which bacteria are transformed with plasmids each containing a different peptide (from a random library); the idea is to obtain thousands of colonies, each containing lipids resulting from the overproduction of a particular peptide. Mass spectrometry and sequencing would then match lipids and peptides. The data would be used to try to derive consensus sequences to be used to interpret the genome and construct a 'lipidome'.

10.4 Atomic Force Microscopy (AFM) and the Langmuir-Blodgett technique

Langmuir-Blodgett monolayers of phospholipids, which assemble at the air-water interface, followed by transfer to a solid support and inspection with AFM, provide a powerful combination of techniques for studying FtsZ interaction with membranes and may constitute the beginnings of an *in vitro* division system (Alexandre *et al*., 2002). The characteristics of the lipids used along with the values of parameters obtained for factors that interact with FtsZ, such as calcium, GTP and other division proteins, might be used to try to construct an *in silico* model of the division process.

11. I-cell

Developing new concepts may prove essential to a full understanding of how a cell works. To test and develop such concepts, we advocate the construction of an *Integrated* or *Imaginary* cell – *I-cell* (Amar *et al*., 2002). An I-cell would undergo selection for growth and survival in a world of artificial chemistry (Demarty *et al*., 2003; Dittrich and Banzhaf, 1998). The unit volumes that constitute an I-cell would be inspected at each time step and, according to the molecule(s) found, the appropriate entry would be consulted in a table containing a large number of 'biological' functions (Norris and Le Sceller, 2001). These functions would determine the interactions of the molecule with its neighbours and also, via global functions, with distant molecules. The I-cell would be fed according to different regimes and, depending on the functions implemented, would grow and eventually divide; I-cells would be analysed after

selection over several generations. Combinatorial problems would be reduced if an activity-based vision of the cell were adopted in which only a subset of constituents would be consulted at each time step; this subset would correspond to constituents that play an active role in coherent cell states via a mechanism based in part on global functions and termed competitive coherence (Norris, 1998). An I-cell might, for example, offer a way to discover the importance of a particular organising process, for example, one based on water structure or tensegrity. An I-cell might even be used to see whether new laws of complexity emerge as the number of organising processes in the system increases.

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